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**Gillespie et al.**

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(54) **DNA ENCODING HUMAN  $\alpha$  AND  $\beta$  SUBUNITS OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR, CELLS TRANSFORMED THEREWITH, AND RECOMBINANT CELL LINE EXPRESSING A HUMAN  $\alpha$  AND  $\beta$  SUBUNIT OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR**

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This patent is subject to a terminal disclaimer.

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**Related U.S. Application Data**

(63) Continuation-in-part of application No. 08/487,596, filed on Jun. 7, 1995, now Pat. No. 6,440,681, which is a continuation-in-part of application No. 08/149,503, filed on Nov. 8, 1993, now abandoned, and a continuation-in-part of application No. 08/028,031, filed on Mar. 8, 1993, now abandoned, and a continuation-in-part of application No. 07/938,154, filed on Nov. 30, 1992, now Pat. No. 5,981,193, which is a continuation-in-part of application No. 07/504,455, filed on Apr. 3, 1990, now Pat. No. 5,369,028.

(51) **Int. Cl.**<sup>7</sup> ..... **C12N 1/20**

(52) **U.S. Cl.** ..... **435/252.3; 435/69.1; 435/320.1; 435/352; 530/350; 536/23.5**

(58) **Field of Search** ..... **435/6, 7.1, 7.21, 435/69.1, 252.3, 320.1, 352; 436/501; 530/350; 514/2; 536/23.1, 23.5**

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(57) **ABSTRACT**

Isolated nucleic acid molecules, i.e., DNA or RNA encoding human neuronal nicotinic acetylcholine receptor alpha and beta subunits, mammalian and amphibian cells containing said DNA, methods for producing  $\alpha$  and  $\beta$  subunits and recombinant (i.e., isolated or substantially pure)  $\alpha$  subunits and  $\beta$  subunits are provided. In addition, cells expressing various multimeric combinations of subunits (i.e.,  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_6$  and/or  $\alpha_7$  in combination with at least one of an  $\alpha$  and  $\beta$  subunit are also provided. A recombinant, non-human cell line expressing the human  $\alpha_7$  subunit of nAChR is disclosed.

**8 Claims, 16 Drawing Sheets**

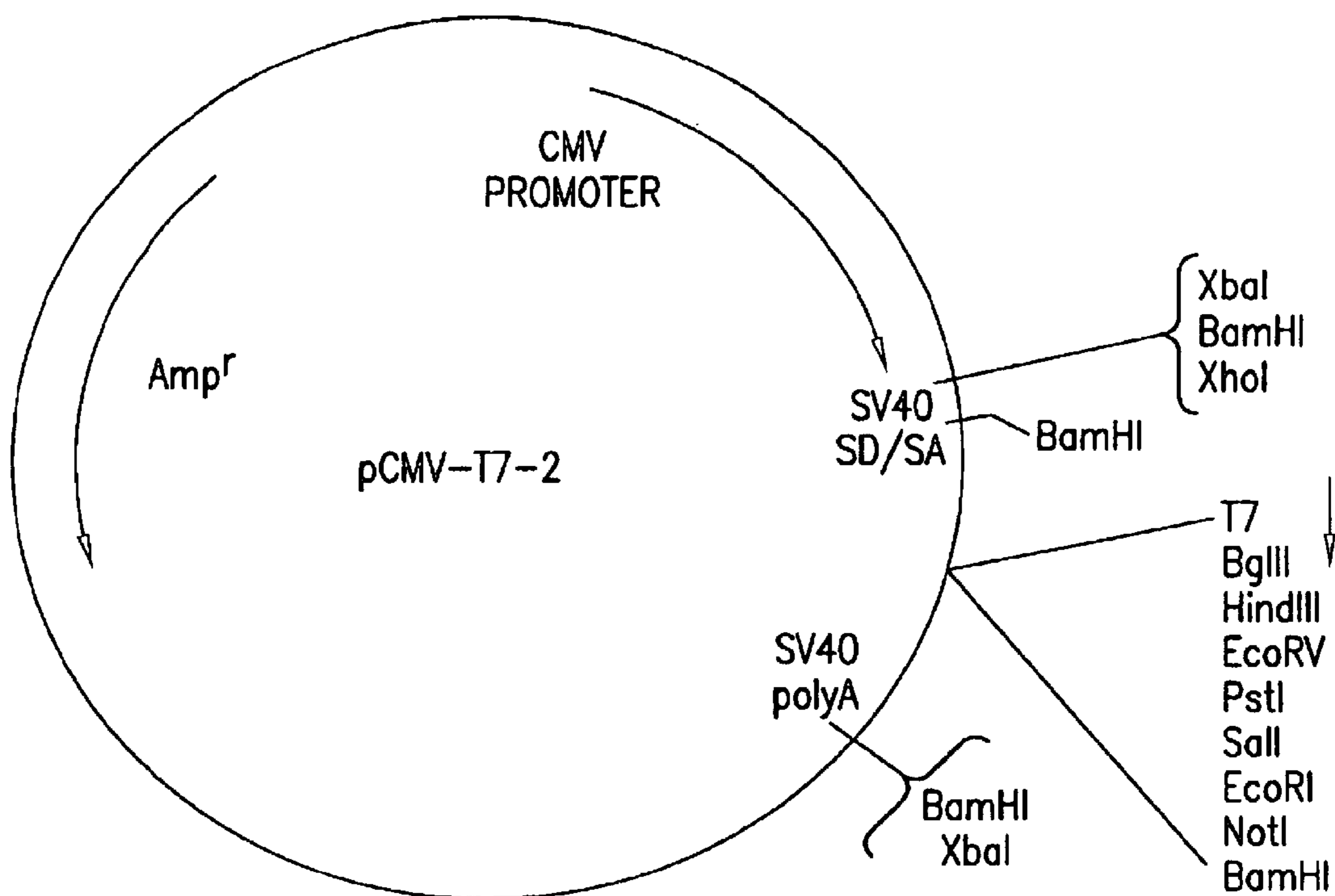


FIG. 1A

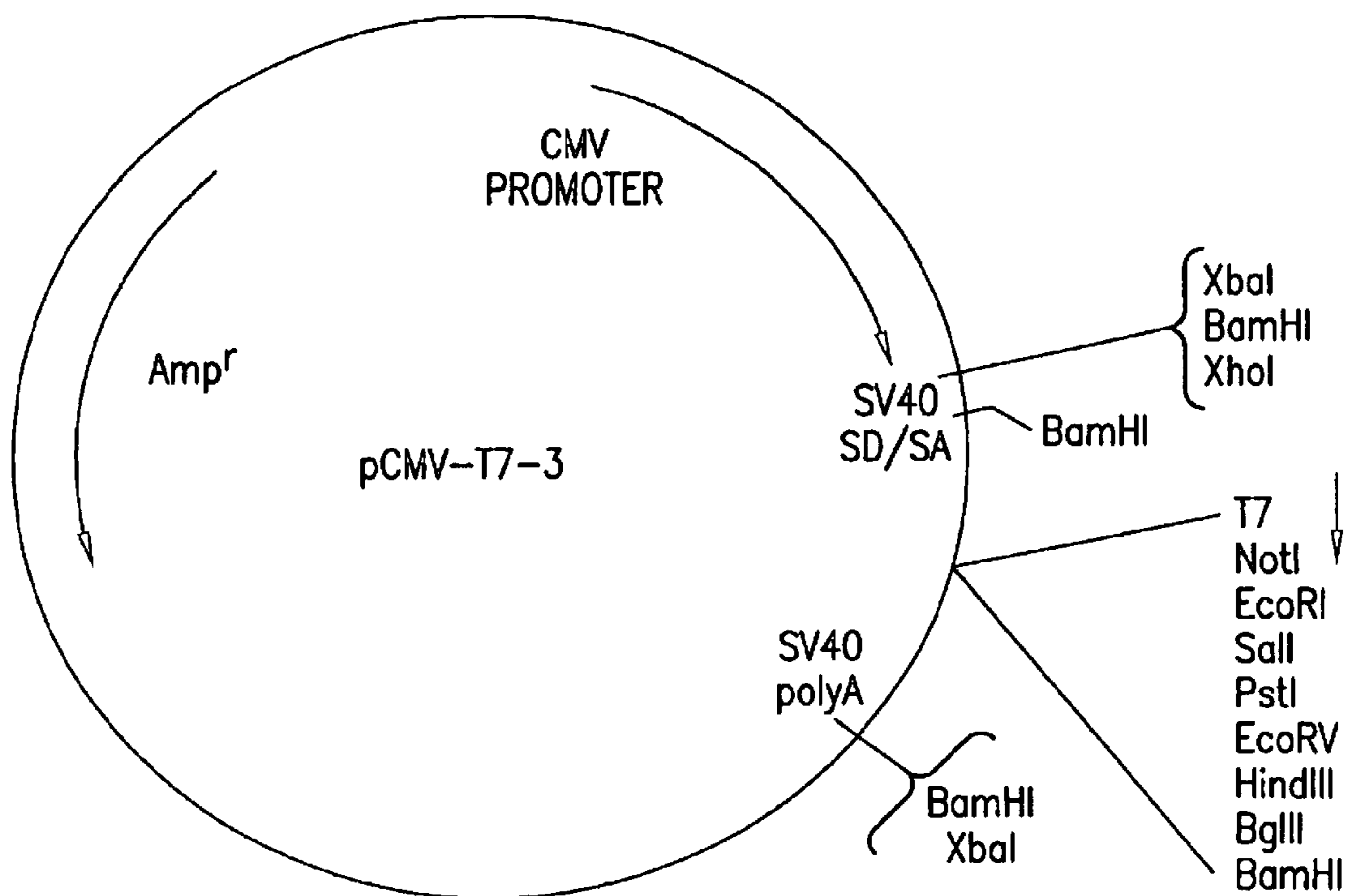


FIG. 1B

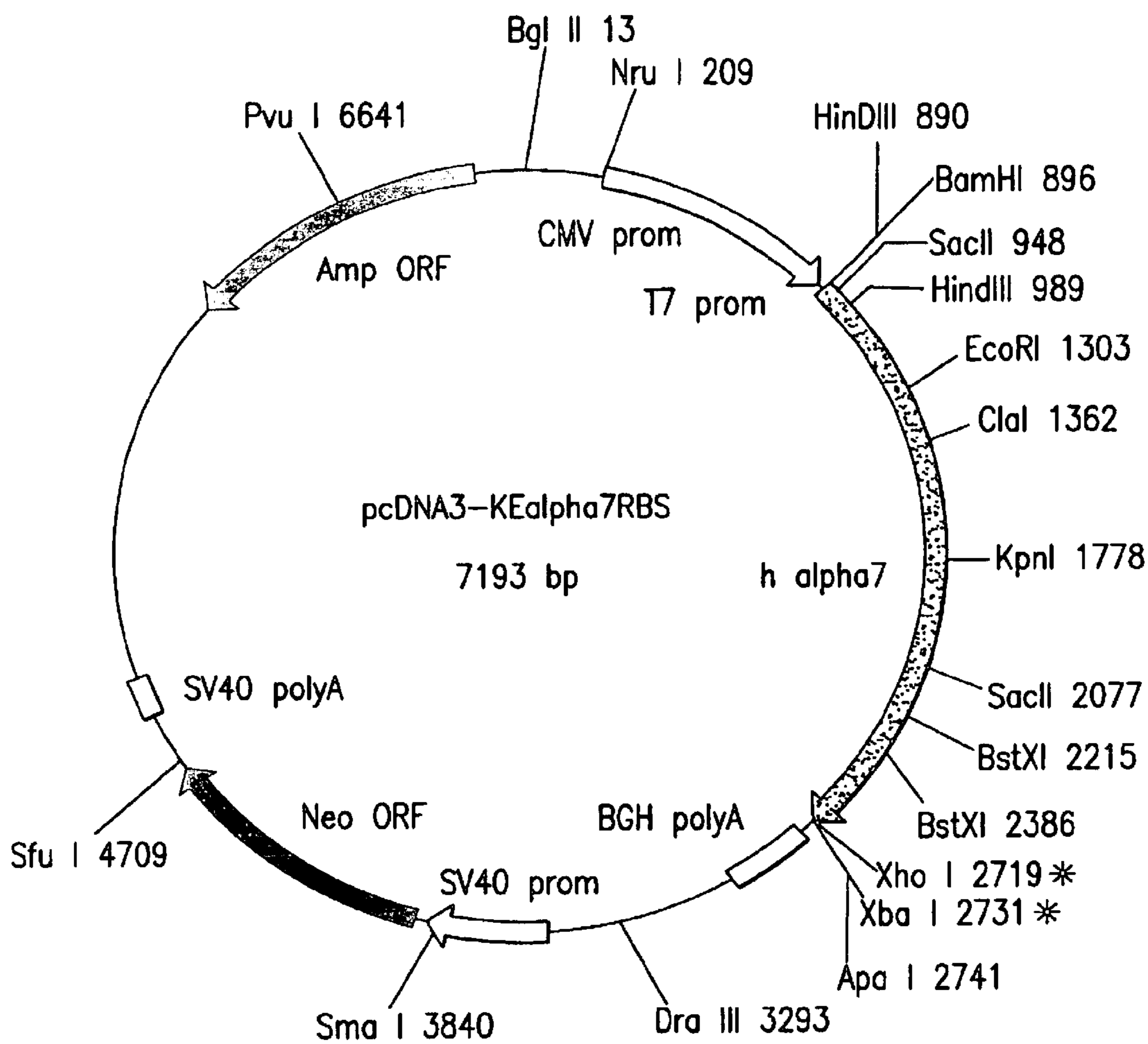


FIG.2

AGONIST-INDUCE INCREASES IN  $[Ca^{2+}]_i$  FOR A7 STABLE  
CELL LINE  
(EXPRESSING THE NICOTINIC ALPHA 7 RECEPTOR IN GH<sub>4</sub>C<sub>1</sub> CELLS)

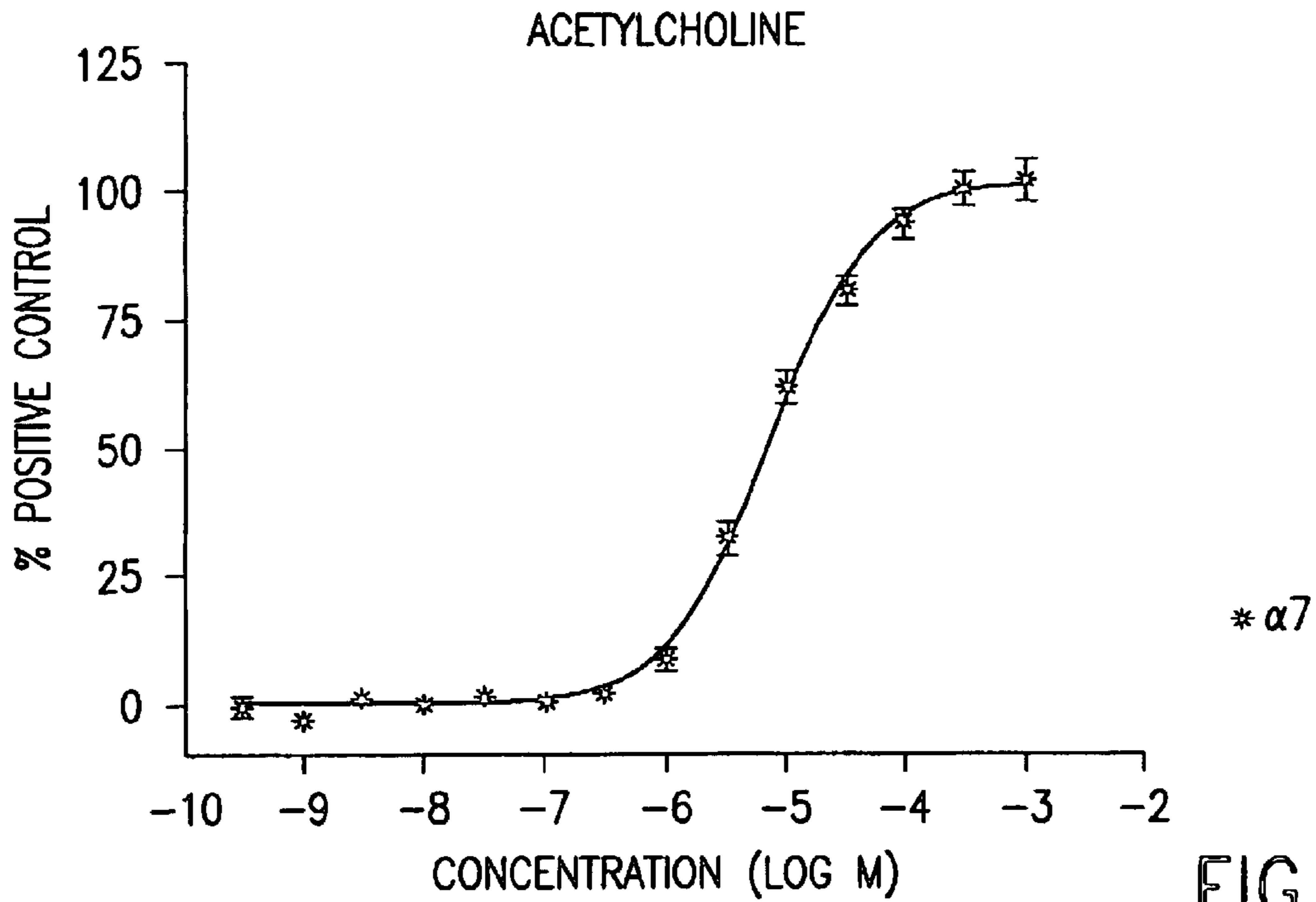


FIG.3A

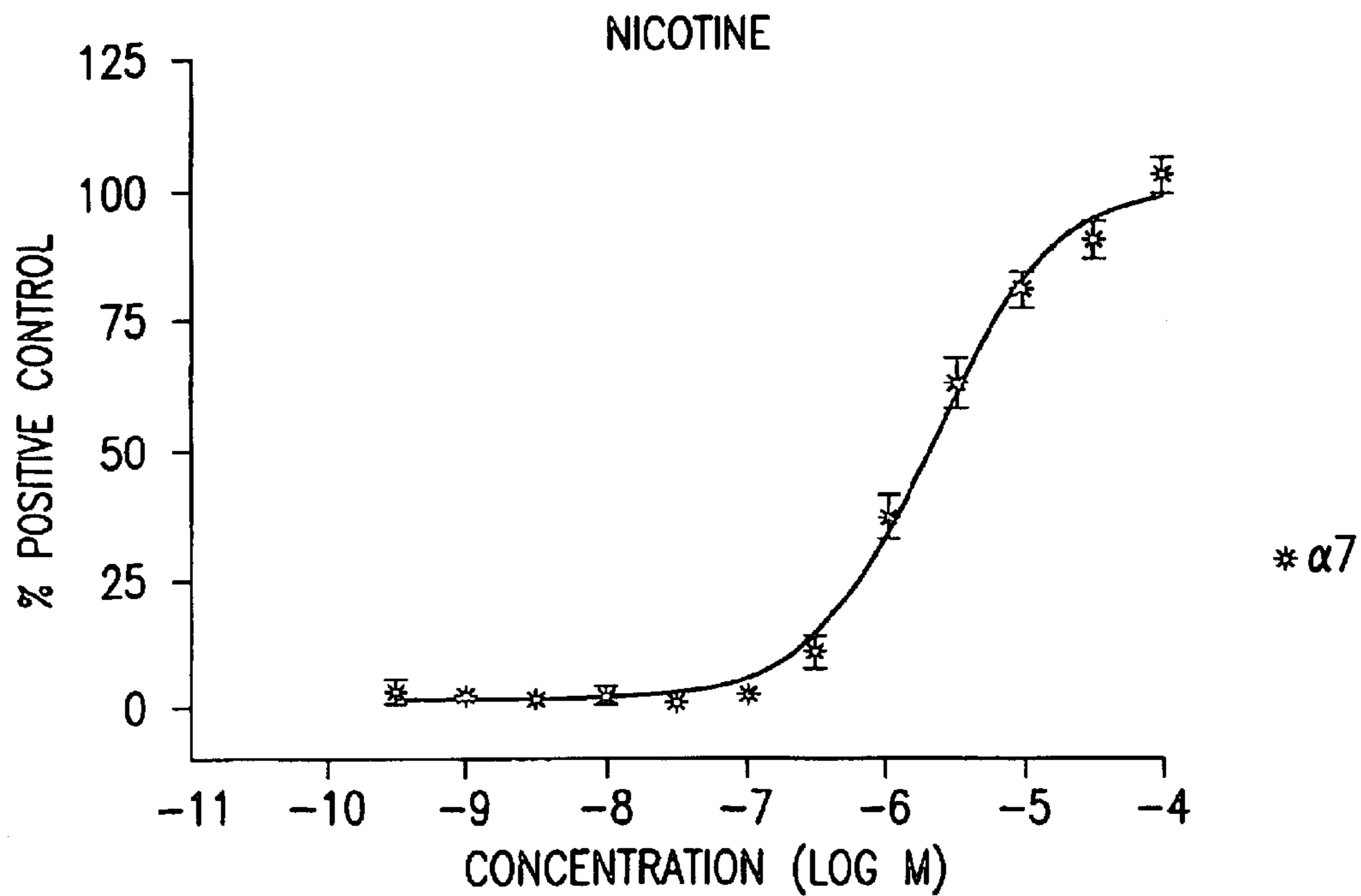


FIG.3B

G1-9-15-8 (A7) CELL

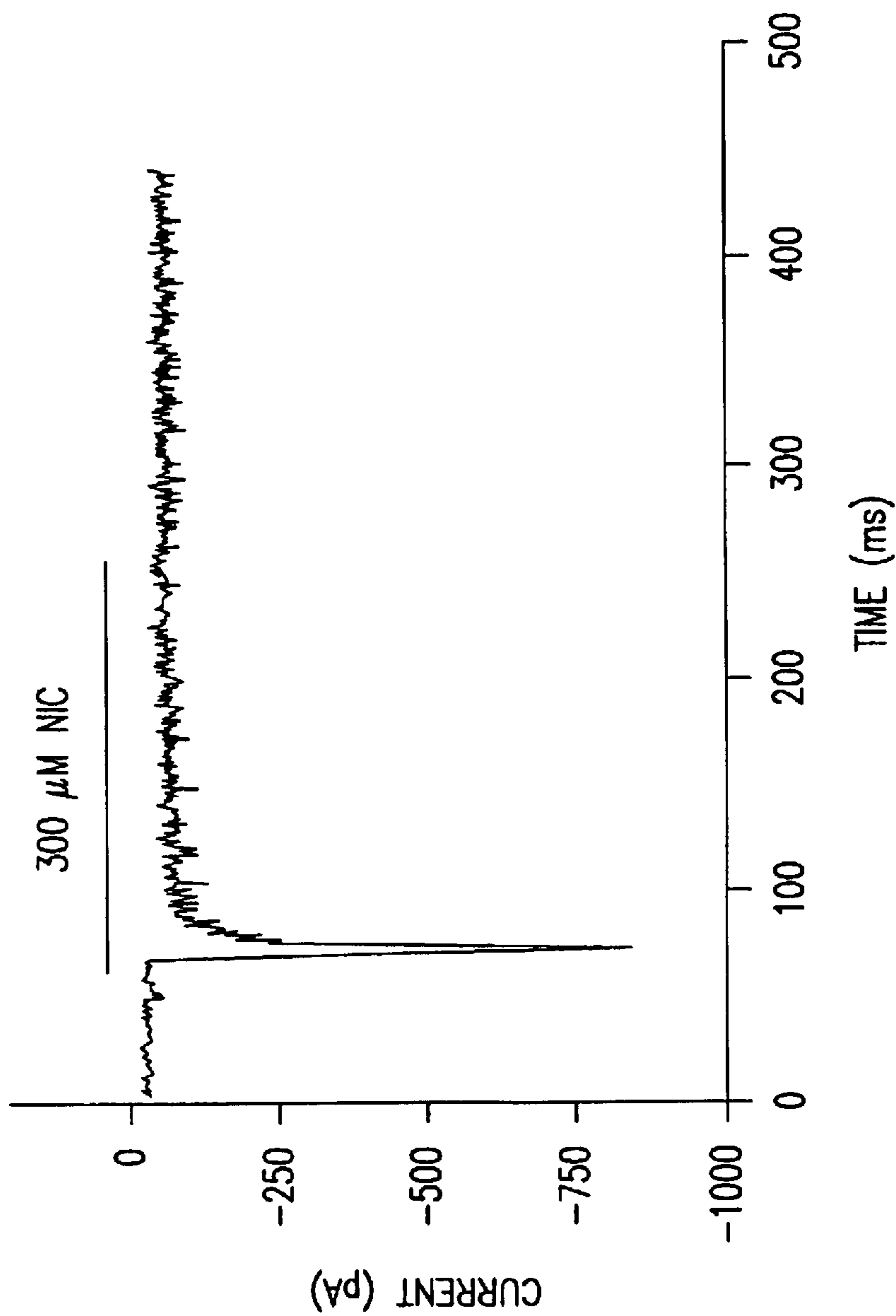
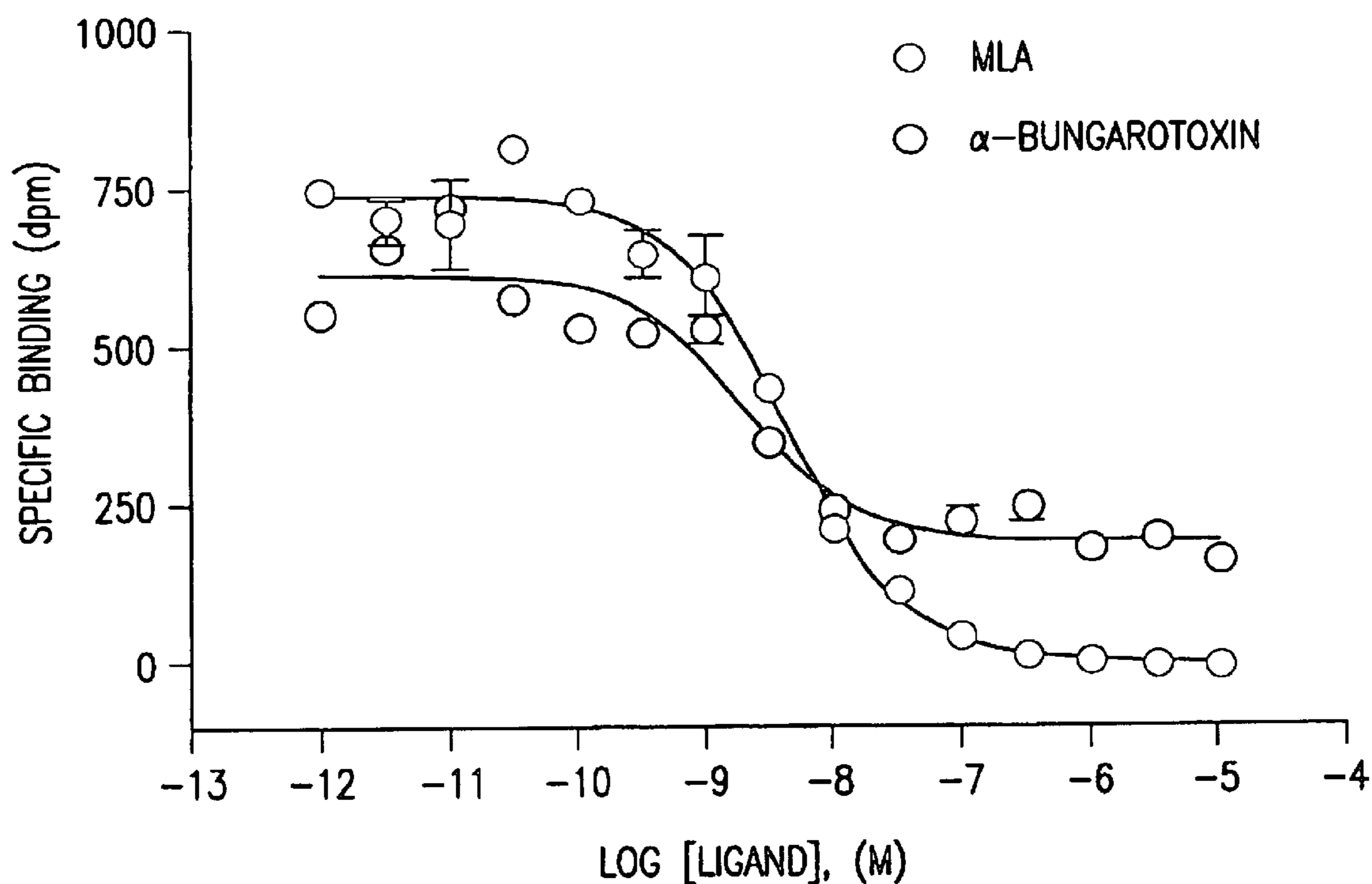


FIG.4

[<sup>3</sup>H]-METHYLLYCACONITINE BINDING TO MEMBRANES PREPARED FROM  $\alpha 7$  EXPRESSED IN GH4C1 WHOLE CELLS

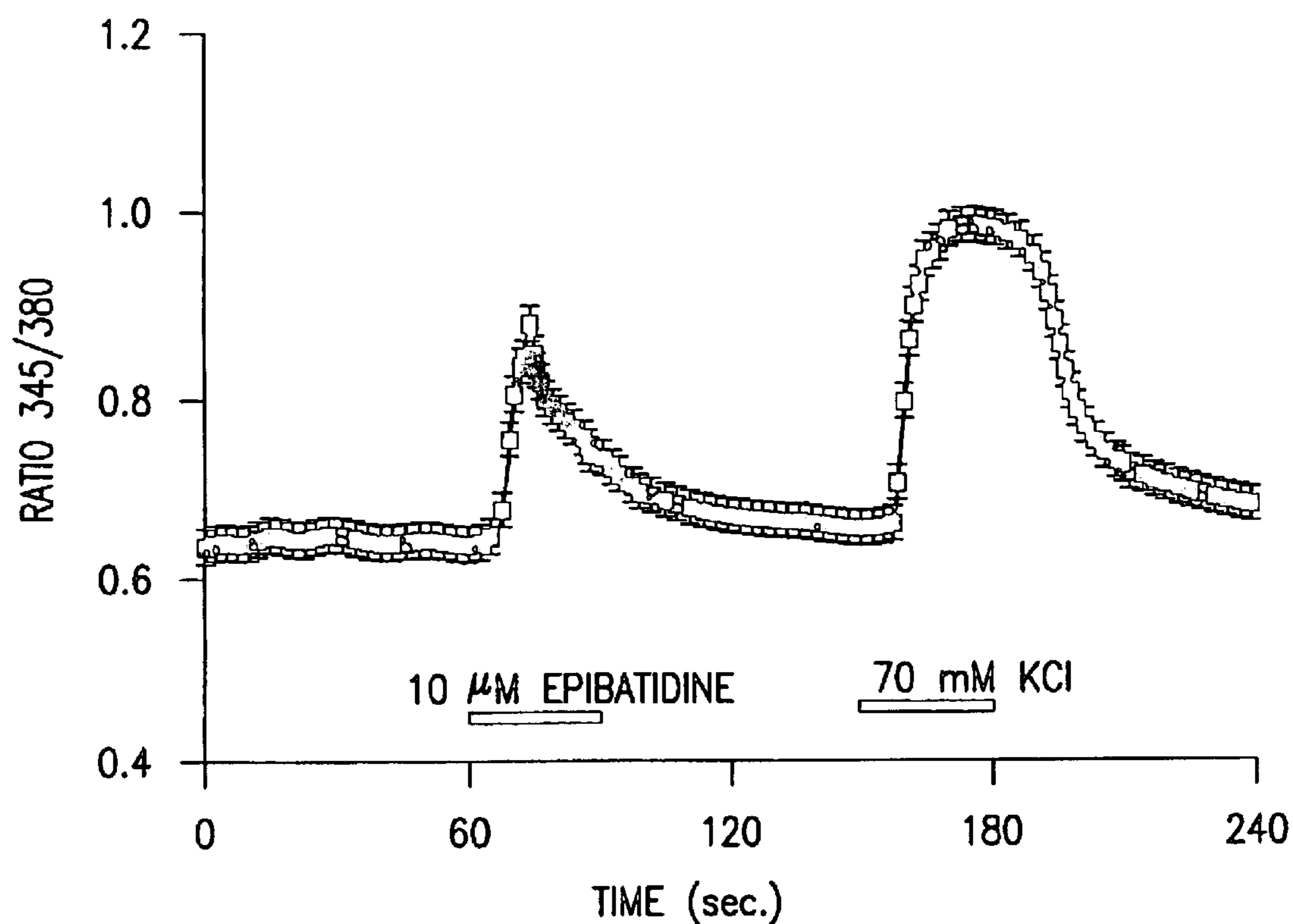


	MLA	$\alpha$ -BUNGAROTOXIN
BOTTOM	0.3228	191.7
TOP	737.1	612.3
LOGEC50	-8.353	-8.689
HILLSLOPE	-0.9527	-0.9856
EC50	4.4320e-009	2.0470e-009

FIG.5

SINGLE-CELL IMAGING DATA DEMONSTRATES  
THE HOMOGENEOUS RESPONSE OF STABLE CELL  
LINE A7 TO EPIBATIDINE

$\alpha_7$  SUBCLONE G1-9-15-8  
15 ROI  $\pm$  SEM



CELLS WERE SUPERFUSED WITH HBS PRIOR TO TREATMENT PERIODS  
AS INDICATED ON THE GRAPH. VALUES ARE MEANS  $\pm$  SEM FROM  
ONE EXPERIMENT. CELLS WERE CULTURED AT 37 °C.

FIG.6

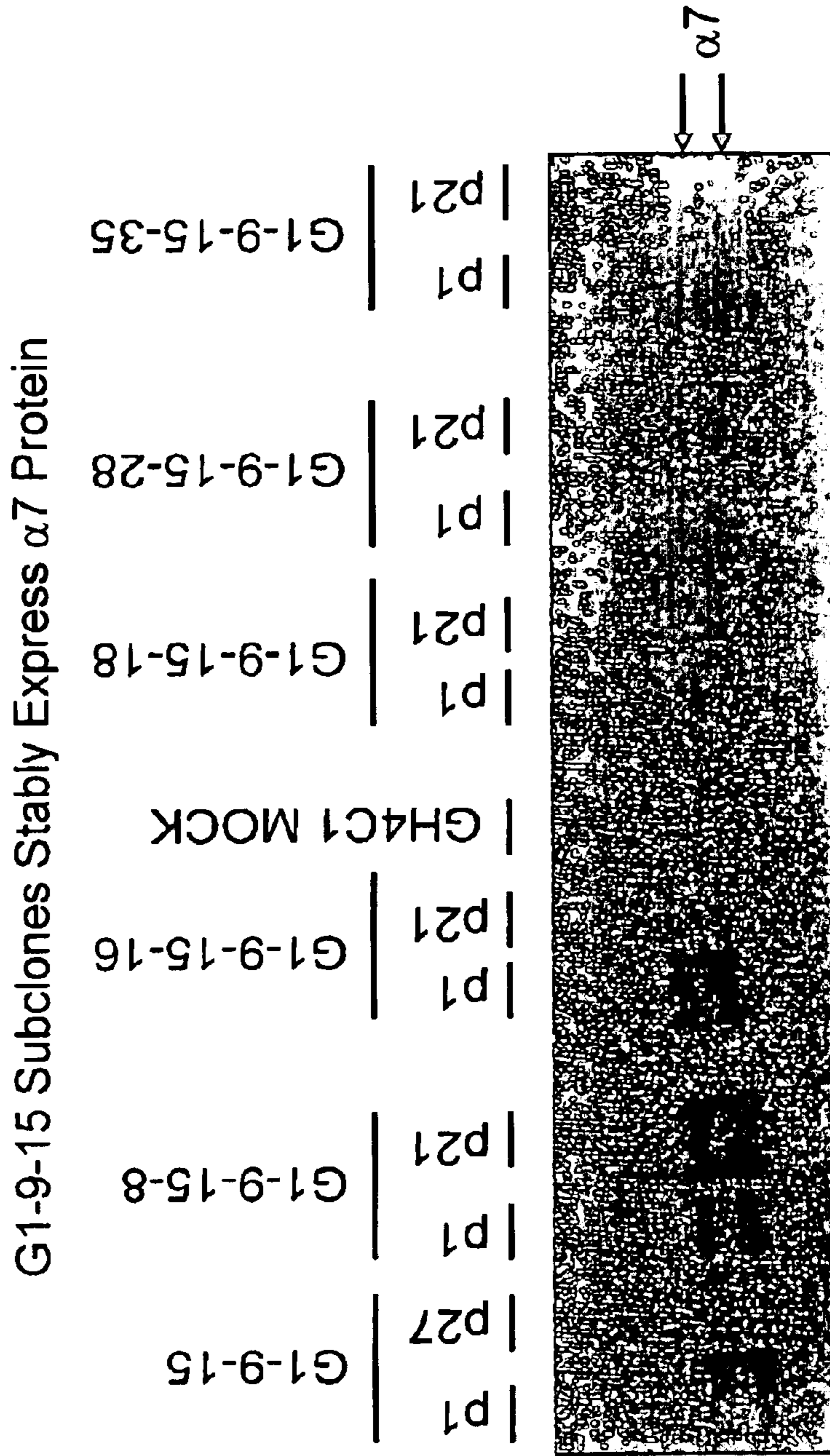


FIG. 7



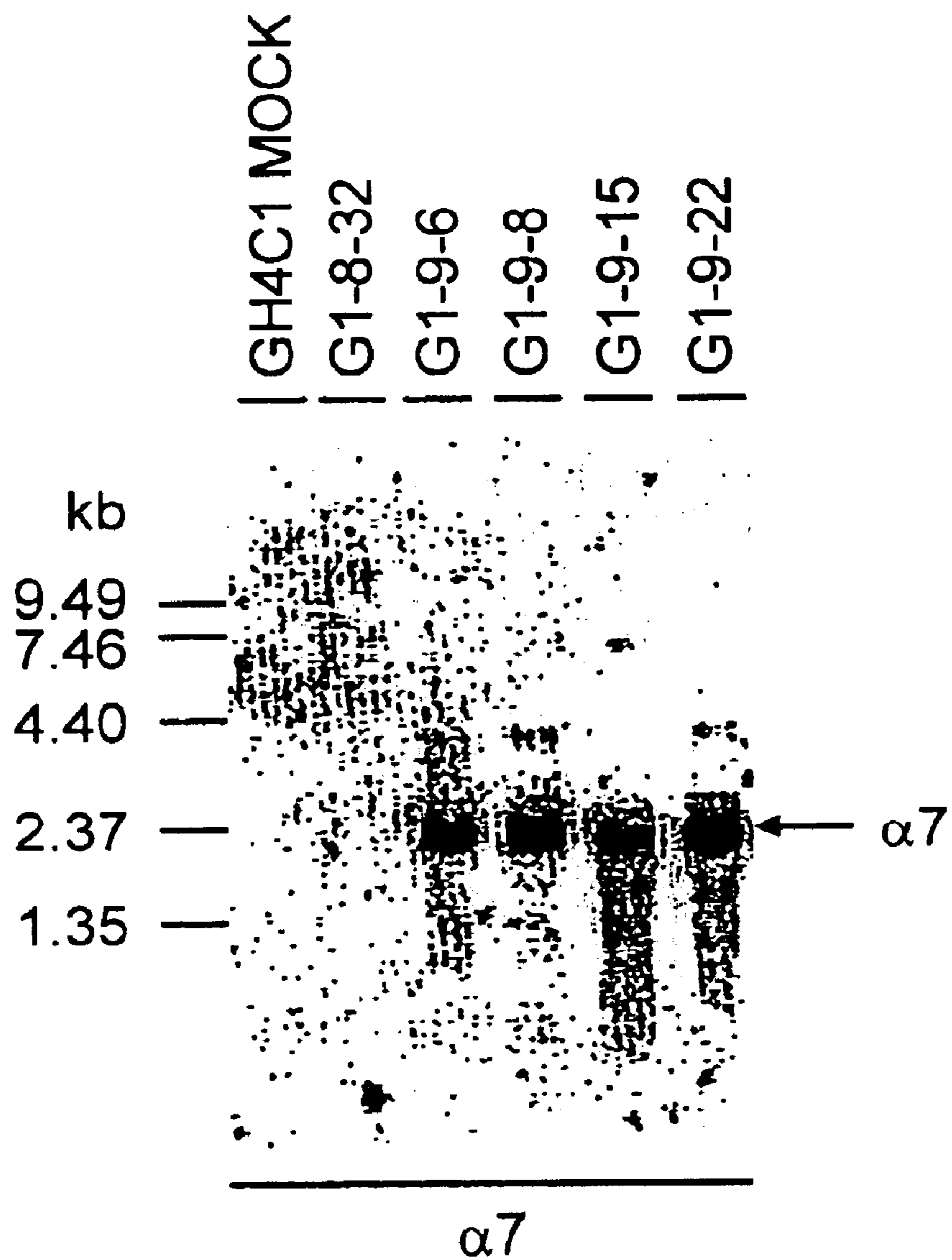


FIG. 8

AGONIST PHARMACOLOGY OF A3B2A5 CELLS  
IS DISTINCT FROM A3B2 ( $\alpha3\beta2$ ) CELLS

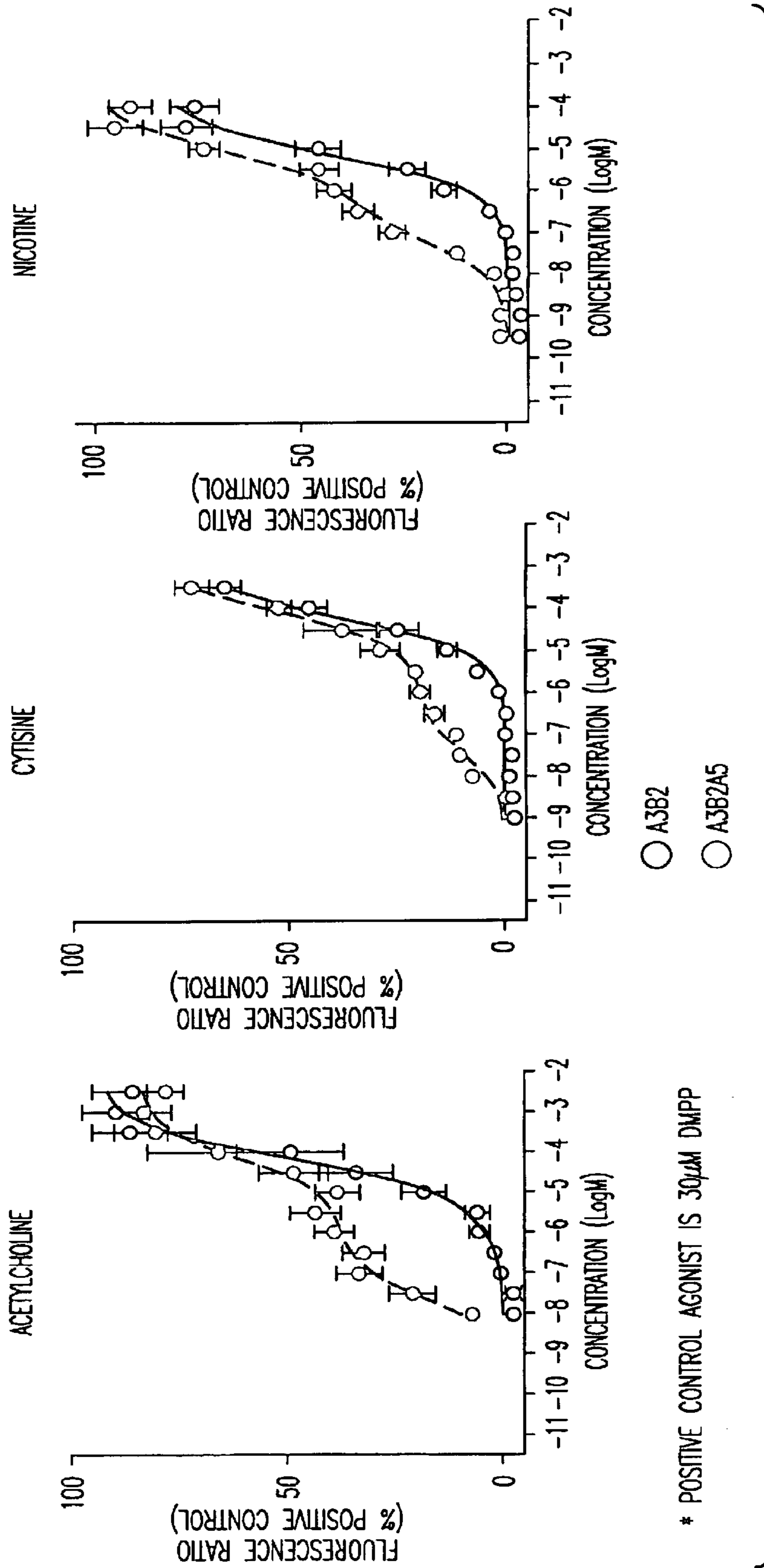


FIG. 9A

AGONIST PHARMACOLOGY OF A3B2A5 CELLS  
IS DISTINCT FROM A3B2 ( $\alpha3\beta2$ ) CELLS

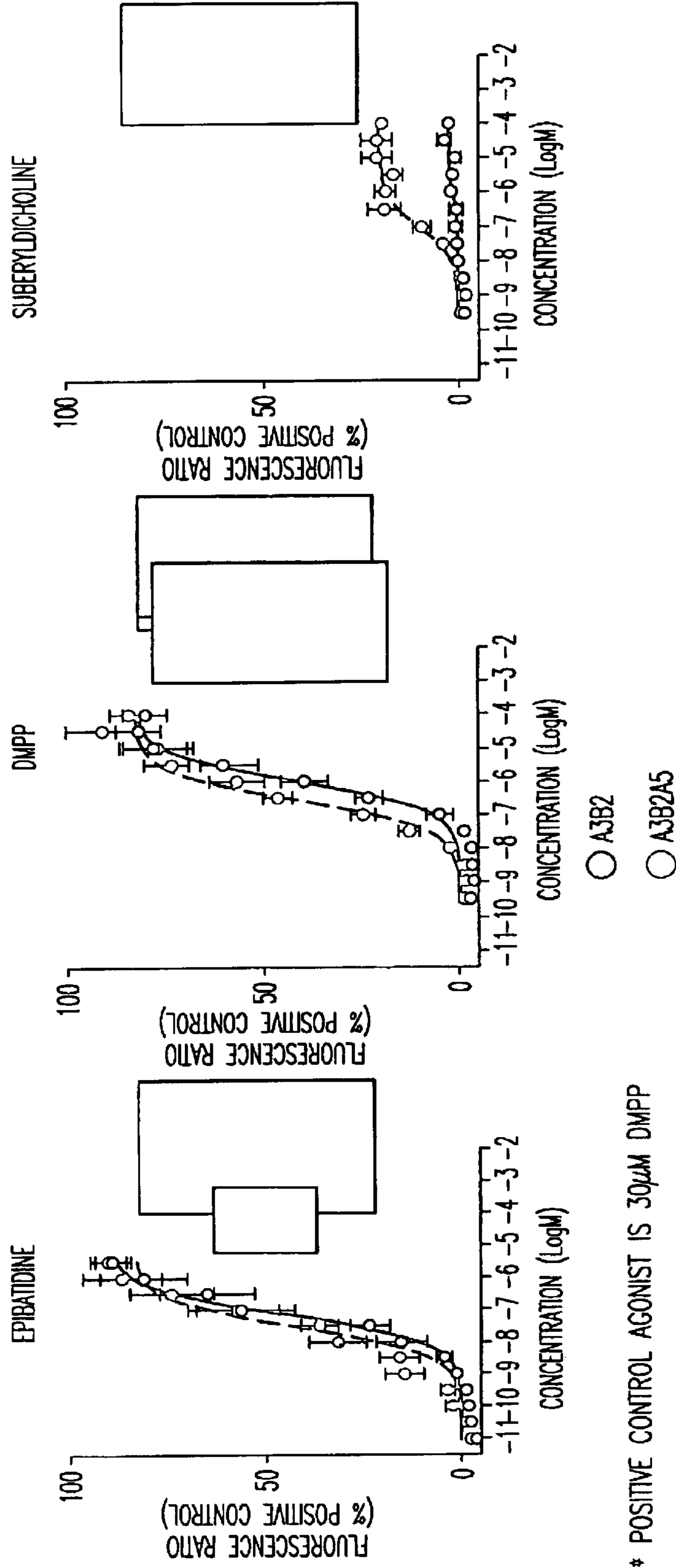


FIG. 9B

THE KINETICS OF DECAY OF CURRENTS INDUCED BY LOW DOSES OF ACETYLCHOLINE ARE SLOWER IN A3B2A5 THAN IN A3B2 CELLS

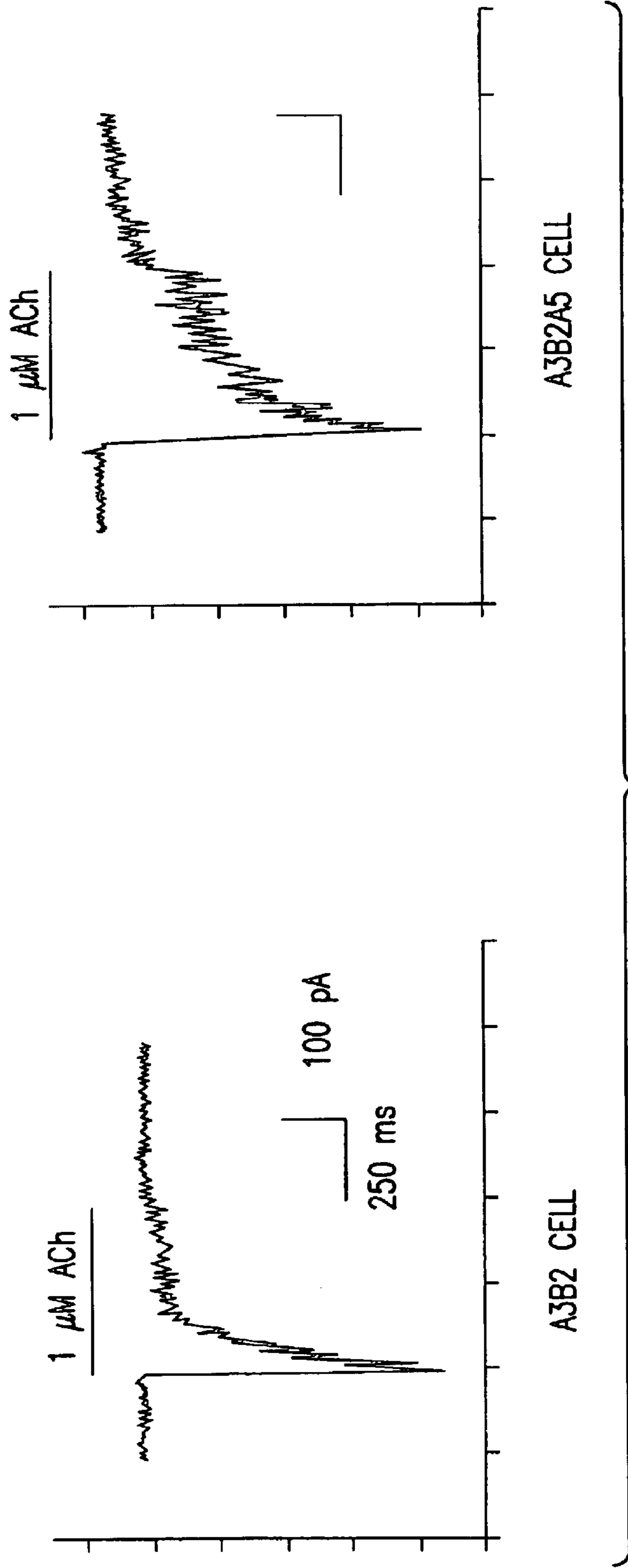
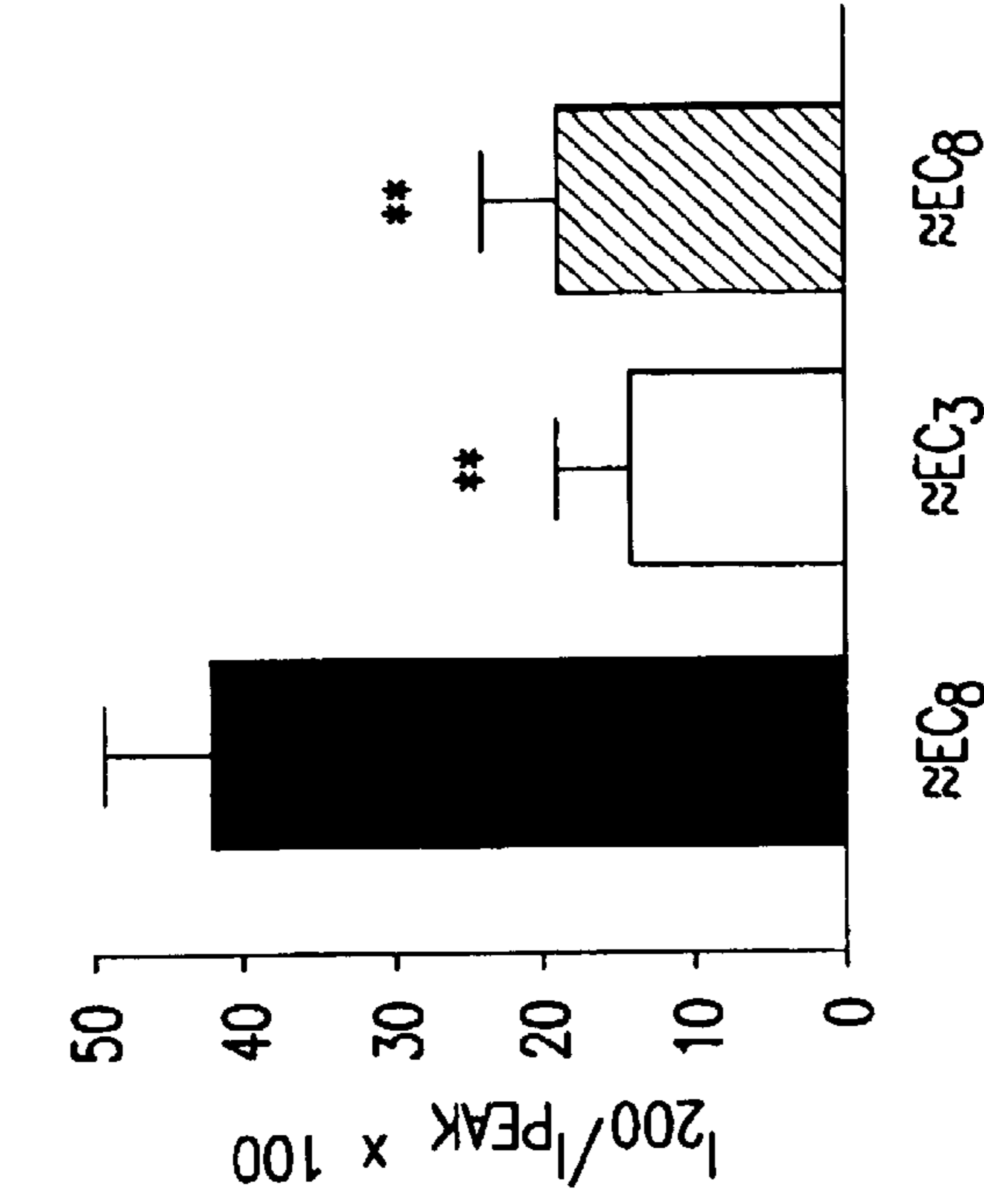


FIG. 10A

RESIDUAL CURRENT MEASURED  
200 ms AFTER CURRENT ONSET



TIME CONSTANT OF DECAY OF  
CURRENTS INDUCED BY ACh

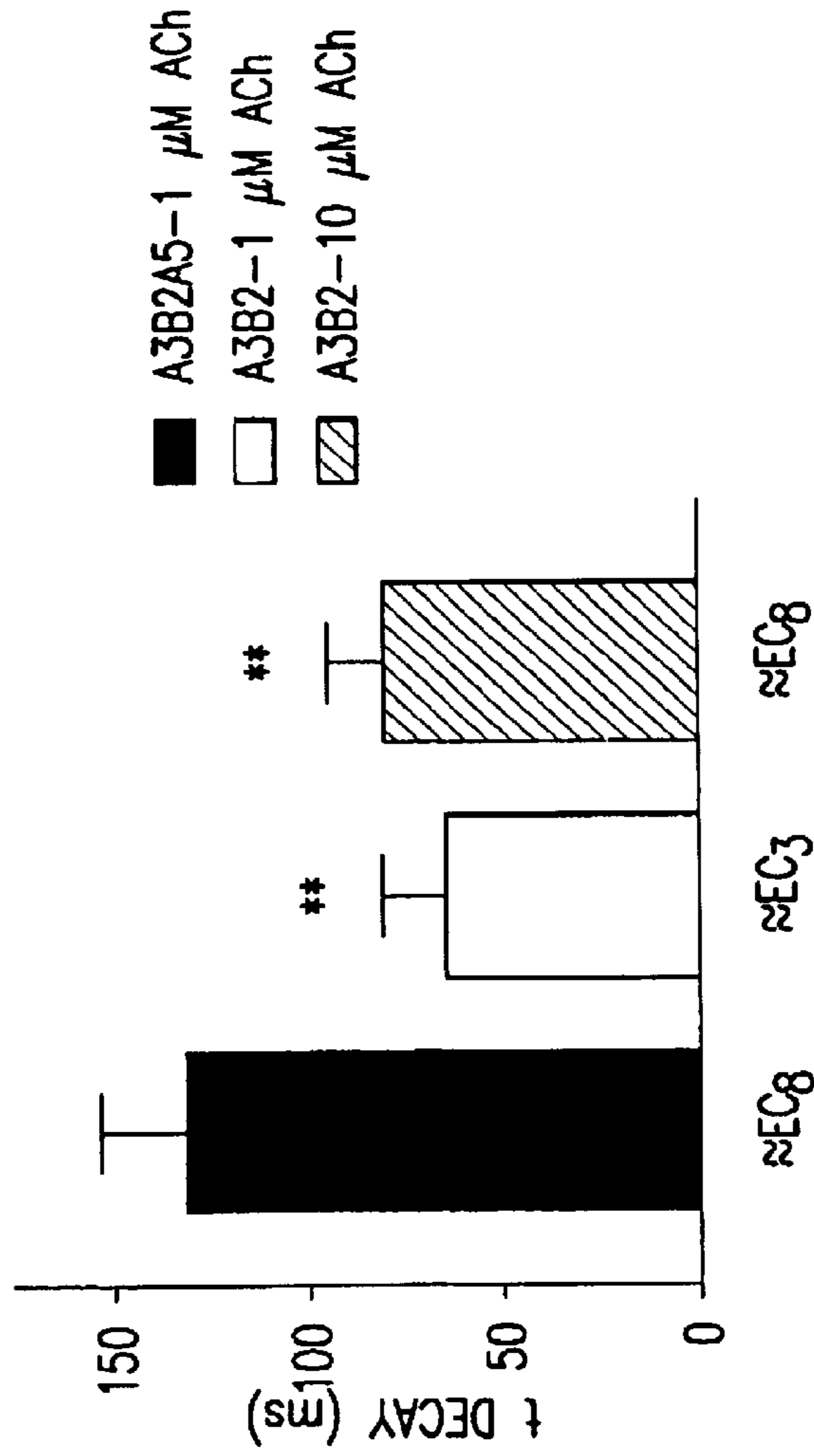


FIG. 10B

$\alpha$ 5 Co-Assembles with  $\alpha$ 3 and  $\beta$ 2 in Cell Line A3B2A5

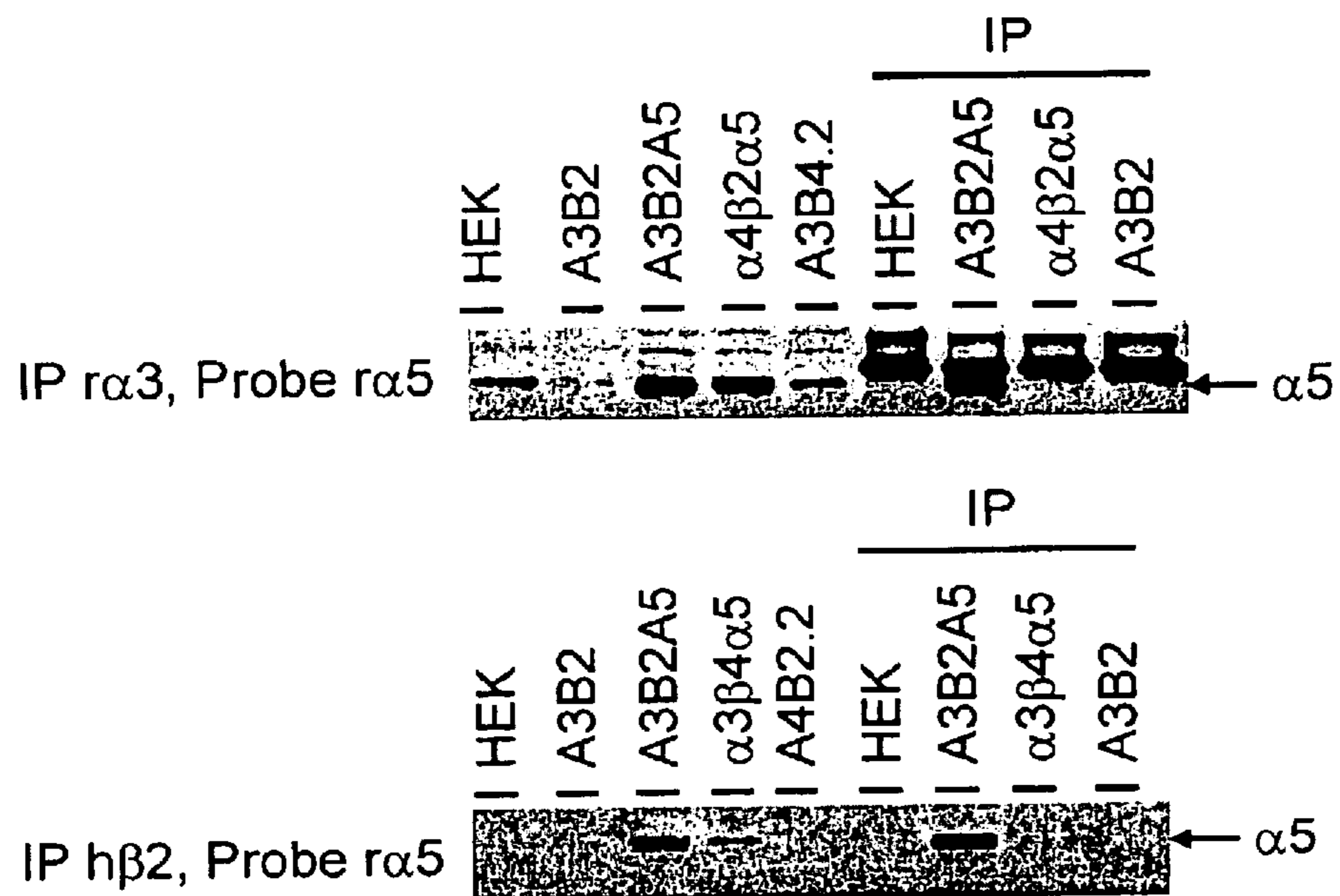


FIG. 11

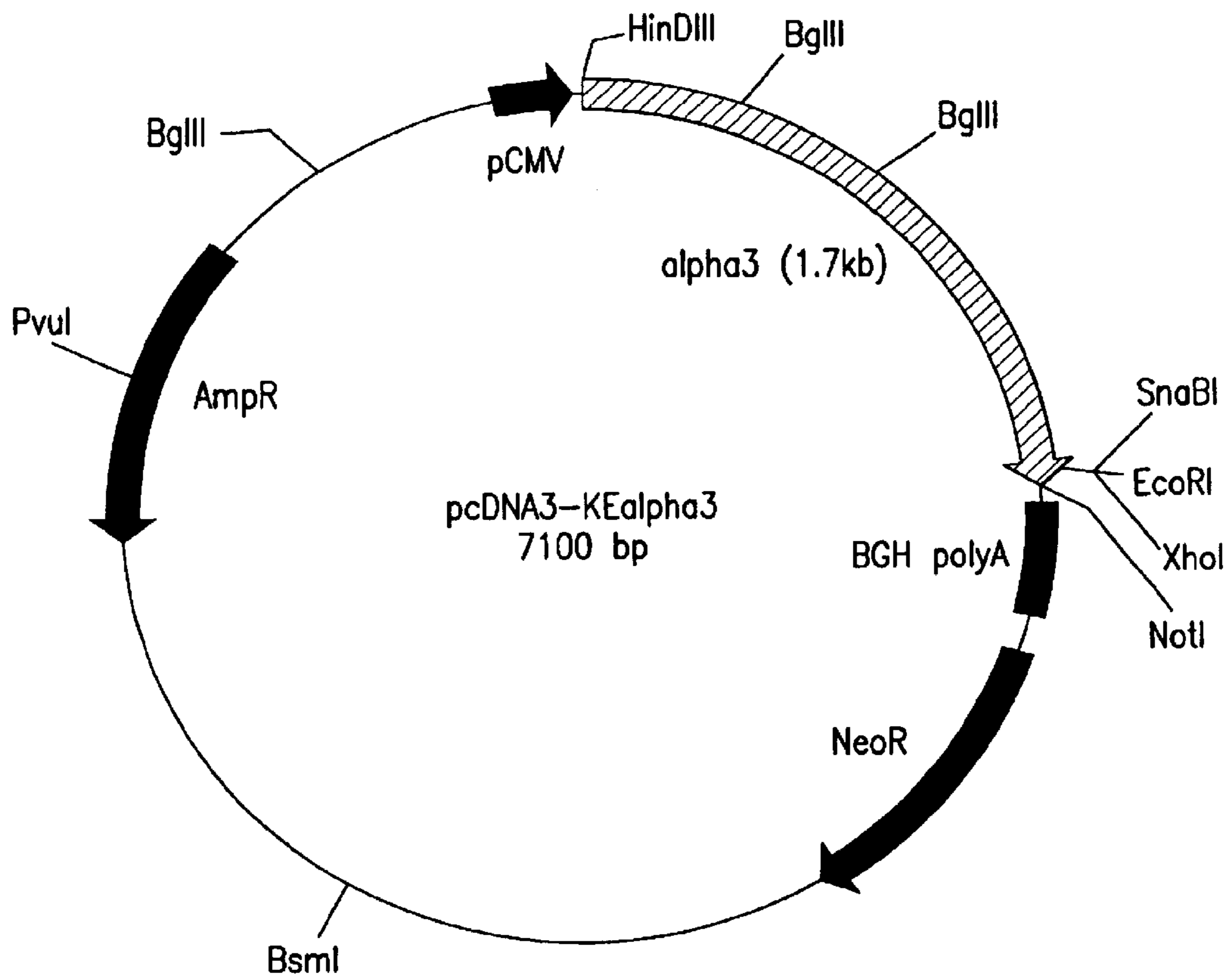


FIG. 12

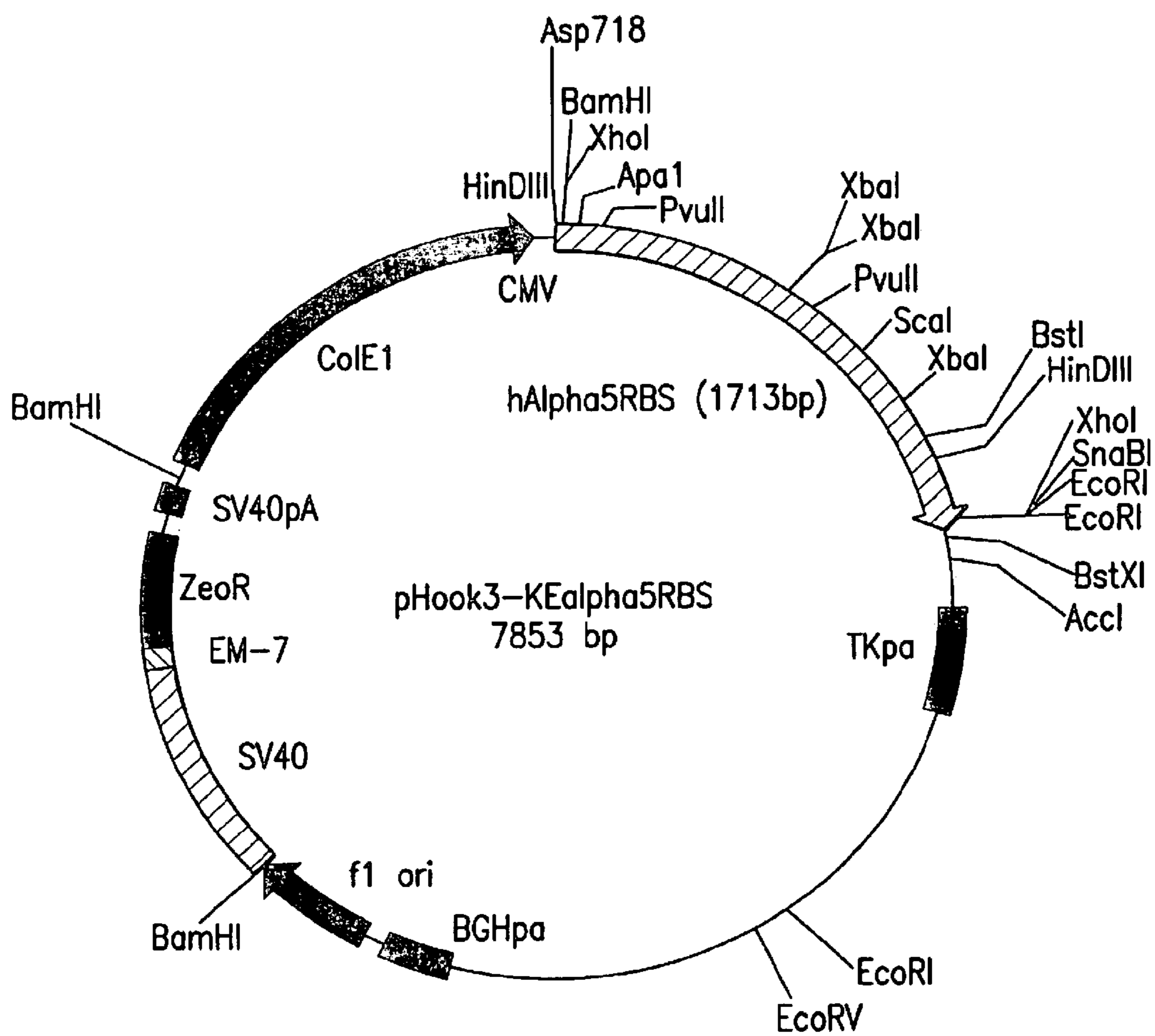


FIG. 13



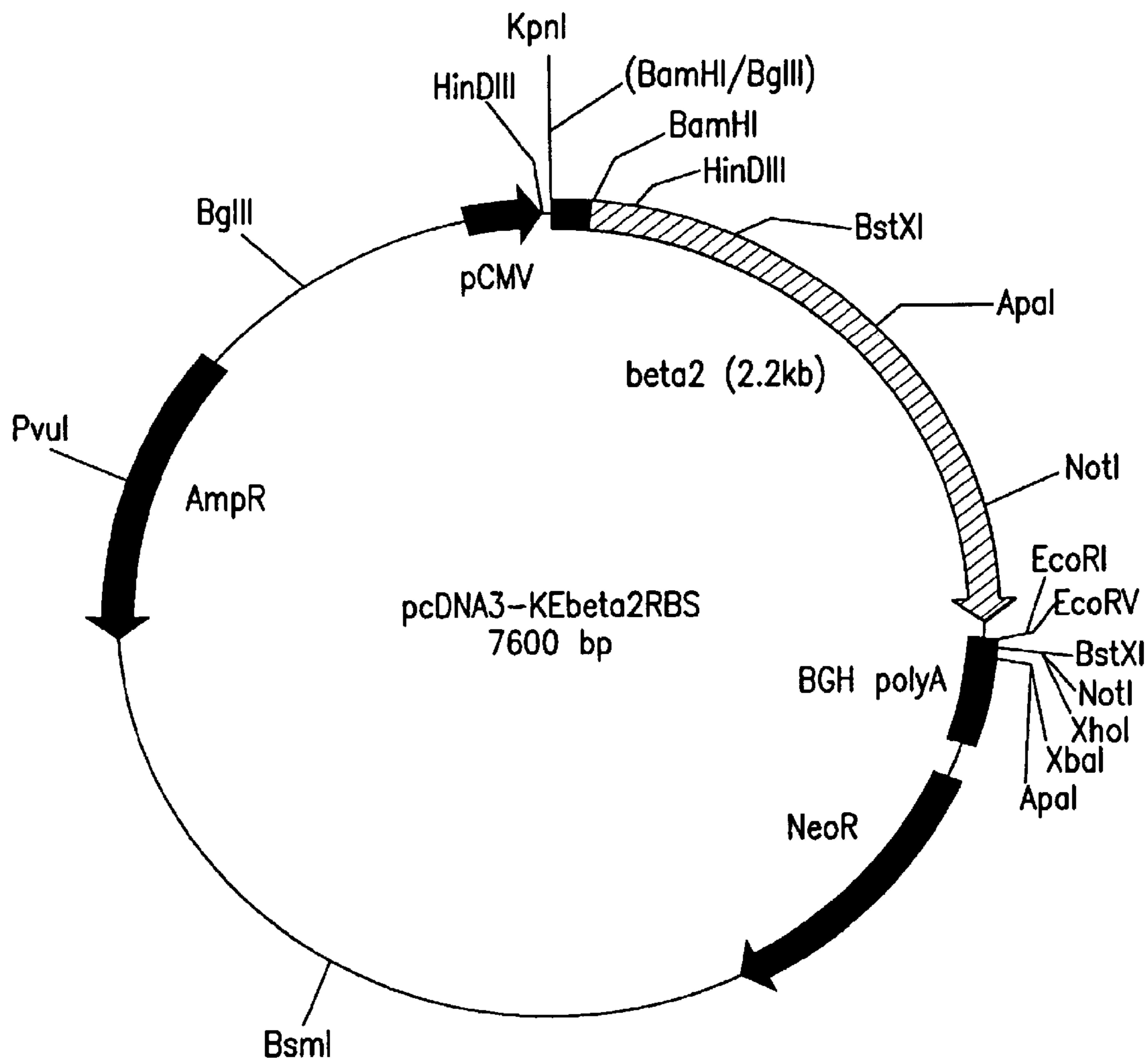


FIG. 14

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**DNA ENCODING HUMAN  $\alpha$  AND  $\beta$   
SUBUNITS OF NEURONAL NICOTINIC  
ACETYLCHOLINE RECEPTOR, CELLS  
TRANSFORMED THEREWITH, AND  
RECOMBINANT CELL LINE EXPRESSING A  
HUMAN  $\alpha$  AND  $\beta$  SUBUNIT OF NEURONAL  
NICOTINIC ACETYLCHOLINE RECEPTOR**

This application is a continuation-in-part of U.S. application Ser. No. 08/487,596 filed Jun. 7, 1995, now issued as U.S. Pat. No. 6,440,681 which is now pending, and which is a continuation-in-part of U.S. application Ser. No. 08/149,503, filed Nov. 8, 1993, now abandoned; and a continuation-in-part of U.S. application Ser. No. 08/028,031, filed Mar. 8, 1993, now abandoned; and a continuation-in-part of U.S. application Ser. No. 07/938,154, filed Nov. 30, 1992, now issued as U.S. Pat. No. 5,981,193, which is a continuation-in-part of U.S. application Ser. No. 07/504,455, filed Apr. 3, 1990, now issued as U.S. Pat. No. 5,369,028, each of which is hereby incorporated by reference herein in their entirety.

This invention relates to DNA encoding human neuronal nicotinic acetylcholine receptor protein subunits, as well as the proteins themselves. In particular, human neuronal nicotinic acetylcholine receptor  $\alpha$ -subunit-encoding DNA,  $\alpha$ -subunit proteins,  $\beta$ -subunit-encoding DNA,  $\beta$ -subunit proteins, and combinations thereof are provided. A non-human cell line expressing a human  $\alpha$ -subunit protein is also disclosed.

#### BACKGROUND OF THE INVENTION

Ligand-gated ion channels provide a means for communication between cells of the central nervous system. These channels convert a signal (e.g., a chemical referred to as a neurotransmitter) that is released by one cell into an electrical signal that propagates along a target cell membrane. A variety of neurotransmitters and neurotransmitter receptors exist in the central and peripheral nervous systems. Five families of ligand-gated receptors, including the nicotinic acetylcholine receptors (NACHRs) of neuromuscular and neuronal origins, have been identified (Stroud et al. (1990) *Biochemistry* 29:11009–11023). There is, however, little understanding of the manner in which the variety of receptors generates different responses to neurotransmitters or to other modulating ligands in different regions of the nervous system.

The nicotinic acetylcholine receptors (NACHRs) are multisubunit proteins of neuromuscular and neuronal origins. These receptors form ligand-gated ion channels that mediate synaptic transmission between nerve and muscle and between neurons upon interaction with the neurotransmitter acetylcholine (ACh). Since various nicotinic acetylcholine receptor (NACHR) subunits exist, a variety of NACHR compositions (i.e., combinations of subunits) exist. The different NACHR compositions exhibit different specificities for various ligands and are thereby pharmacologically distinguishable. Thus, the nicotinic acetylcholine receptors expressed at the vertebrate neuromuscular junction in vertebrate sympathetic ganglia and in the vertebrate central nervous system have been distinguished on the basis of the effects of various ligands that bind to different NACHR compositions. For example, the elapid  $\alpha$ -neurotoxins that block activation of nicotinic acetylcholine receptors at the neuromuscular junction do not block activation of some neuronal nicotinic acetylcholine receptors that are expressed on several different neuron-derived cell lines.

Muscle NACHR is a glycoprotein composed of five subunits with the stoichiometry  $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$ . Each of the

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subunits has a mass of about 50–60 kilodaltons (kd) and is encoded by a different gene. The  $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$  complex forms functional receptors containing two ligand binding sites and a ligand-gated transmembrane channel. Upon interaction with a cholinergic agonist, muscle nicotinic AChRs conduct sodium ions. The influx of sodium ions rapidly short-circuits the normal ionic gradient maintained across the plasma membrane, thereby depolarizing the membrane. By reducing the potential difference across the membrane, a chemical signal is transduced into an electrical signal that signals muscle contraction at the neuromuscular junction.

Functional muscle nicotinic acetylcholine receptors have been formed with  $\alpha\beta\delta\gamma$  subunits,  $\alpha\beta\gamma$  subunits,  $\alpha\beta\delta$  subunits,  $\beta\delta\gamma$  subunits or  $\alpha\delta$  subunits, but not with only one subunit (see e.g., Kurosaki et al. (1987) *FEBS Lett.* 214:253–258; Camacho et al. (1993) *J. Neuroscience* 13:605–613). In contrast, functional neuronal AChRs (nAChRs) can be formed from  $\alpha$  subunits alone or combinations of  $\alpha$  and  $\beta$  subunits. The larger  $\alpha$  subunit is generally believed to be the ACh-binding subunit and the lower molecular weight  $\beta$  subunit is generally believed to be the structural subunit, although it has not been definitively demonstrated that the  $\beta$  subunit does not have the ability to bind ACh. Each of the subunits which participate in the formation of a functional ion channel are, to the extent they contribute to the structure of the resulting channel, “structural” subunits, regardless of their ability (or inability) to bind ACh. Neuronal AChRs (nAChRs), which are also ligand-gated ion channels, are expressed in ganglia of the autonomic nervous system and in the central nervous system (where they mediate signal transmission), in post-synaptic locations (where they modulate transmission), and in pre- and extra-synaptic locations (where they may have additional functions).

DNA encoding NACHRs has been isolated from several sources. Based on the information available from such work, it has been evident for some time that NACHRs expressed in muscle, in autonomic ganglia, and in the central nervous system are functionally diverse. This functional diversity could be due, at least in part, to the large number of different NACHR subunits which exist. There is an incomplete understanding, however, of how (and which) NACHR subunits combine to generate unique NACHR subtypes, particularly in neuronal cells. Indeed, there is evidence that only certain NACHR subtypes may be involved in diseases such as Alzheimer’s disease. Moreover, it is not clear whether NACHRs from analogous tissues or cell types are similar across species.

Accordingly, there is a need for the isolation and characterization of DNAs encoding each human neuronal NACHR subunit, recombinant cells containing such subunits and receptors prepared therefrom. In order to study the function of human neuronal AChRs and to obtain disease-specific pharmacologically active agents, there is also a need to obtain isolated (preferably purified) human neuronal nicotinic AChRs, and isolated (preferably purified) human neuronal nicotinic AChR subunits. In addition, there is also a need to develop assays to identify such pharmacologically active agents.

The availability of such DNAs, cells, receptor subunits and receptor compositions will eliminate the uncertainty of speculating as to human nNACHR structure and function based on predictions drawn from non-human nNACHR data, or human or non-human muscle or ganglia NACHR data.

Therefore, it is an object herein to isolate and characterize DNA encoding subunits of human neuronal nicotinic ace-

tylcholine receptors. It is also an object herein to provide methods for recombinant production of human neuronal nicotinic acetylcholine receptor subunits. It is also an object herein to provide purified receptor subunits and to provide methods for screening compounds to identify compounds that modulate the activity of human neuronal AChRs.

Likewise, it is an object of the present invention to provide a recombinant non-human cell line transformed with a heterologous nucleic acid molecule that encodes a human  $\alpha$  subunit of neuronal nAChR.

These and other objects will become apparent to those of skill in the art upon further study of the specification and claims.

### BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided isolated DNAs encoding novel human alpha and beta subunits of neuronal NACHRs. Also provided is a non-human cell line that expresses a human  $\alpha_7$  subunit of neuronal nAChR. In particular, isolated DNA encoding human  $\alpha_4$ ,  $\alpha_7$ , and  $\beta_4$  subunits of neuronal NACHRs are provided. Messenger RNA and polypeptides encoded by the above-described DNA are also provided.

Further in accordance with the present invention, there are provided recombinant human neuronal nicotinic AChR subunits, including  $\alpha_4$ ,  $\alpha_7$ , and  $\beta_4$  subunits, as well as methods for the production thereof. In addition, recombinant human neuronal nicotinic acetylcholine receptors containing at least one human neuronal nicotinic AChR subunit are also provided, as well as methods for the production thereof. Further provided are recombinant neuronal nicotinic AChRs that contain a mixture of one or more NACHR subunits encoded by a host cell, and one or more nAChR subunits encoded by heterologous DNA or RNA (i.e., DNA or RNA as described herein that has been introduced into the host cell), as well as methods for the production thereof.

Plasmids containing DNA encoding the above-described subunits are also provided. Recombinant cells containing the above-described DNA, mRNA or plasmids are also provided herein. Such cells are useful, for example, for replicating DNA, for producing human NACHR subunits and recombinant receptors, and for producing cells that express receptors containing one or more human subunits.

Also provided in accordance with the present invention are methods for identifying cells that express functional nicotinic acetylcholine receptors. Methods for identifying compounds which modulate the activity of NACHRs are also provided. Invention methods employ that isolated DNAs, encoding human  $\alpha$  and  $\beta$  subunits of neuronal AChRs and polypeptides encoded thereby.

The DNA, mRNA, vectors, receptor subunits, receptor subunit combinations and cells provided herein permit production of selected neuronal nicotinic AChR receptor subtypes and specific combinations thereof, as well as antibodies to said receptor subunits. This provides a means to prepare synthetic or recombinant receptors and receptor subunits that are substantially free of contamination from many other receptor proteins whose presence can interfere with analysis of a single NACHR subtype. The availability of desired receptor subtypes makes it possible to observe the effect of a drug substance on a particular receptor subtype and to thereby perform initial in vitro screening of the drug substance in a test system that is specific for humans and specific for a human neuronal nicotinic AChR subtype.

The availability of subunit-specific antibodies makes possible the application of the technique of immunohistochem-

istry to monitor the distribution and expression density of various subunits (e.g., in normal vs diseased brain tissue). Such antibodies could also be employed for diagnostic and therapeutic applications.

The ability to screen drug substances in vitro to determine the effect of the drug on specific receptor compositions should permit the development and screening of receptor subtype-specific or disease-specific drugs. Also, testing of single receptor subunits or specific receptor subtype combinations with a variety of potential agonists or antagonists provides additional information with respect to the function and activity of the individual subunits and should lead to the identification and design of compounds that are capable of very specific interaction with one or more of the receptor subunits or receptor subtypes. The resulting drugs should exhibit fewer unwanted side effects than drugs identified by screening with cells that express a variety of subtypes.

Further in relation to drug development and therapeutic treatment of various disease states, the availability of DNAs encoding human nAChR subunits enables identification of any alterations in such genes (e.g., mutations) which may correlate with the occurrence of certain disease states. In addition, the creation of animal models of such disease states becomes possible, by specifically introducing such mutations into synthetic DNA sequences which can then be introduced into laboratory animals or in vitro assay systems to determine the effects thereof.

### DETAILED DESCRIPTION OF THE FIGURES

FIG. 1 presents a restriction map of two pCMV promoter-based vectors, pCMV-T7-2 and pCMV-T7-3.

FIG. 2 presents a restriction map of a pCMV promoter based vector, pcDNA3-KE $\alpha_7$ RBS.

FIG. 3 depicts the nicotine and acetylcholine-induced dose-response curves for the A7 cell line obtained from functional bulk calcium assays.

FIG. 4 depicts the kinetics of the A7 stable cell line obtained by electrophysiological analysis.

FIG. 5 depicts the MLA and  $\alpha$ -bungarotoxin (ligands of A7) binding assay of A7.

FIG. 6 depicts the results of a single cell calcium imaging of the A7 cell line, showing the homogeneity of the response of the A7 cell line to acetylcholine.

FIG. 7 depicts the results of a Western blot analysis using an A-7 specific antibody as a probe. The data specifically confirm expression of the  $\alpha_7$  protein by the A7 cells.

FIG. 8 shows the results of a Northern Blot analysis of total RNA prepared from A7 cells.

FIGS. 9a-b compares agonist-induced dose-response curves of the alpha3beta2alpha5 expressing cells and alpha3beta2 expressing cells and specifically shows that their profile differs from that of an alpha3beta2 subunit combination.

FIG. 10 depicts a comparison in the kinetics of decay of currents induced by acetylcholine between A3B2A5 cells and A3B2 cells.

FIG. 11 confirms the association of the alpha3 and beta 2 with alpha 5 subunits in cell line A3B2A5.

FIG. 12 depicts the expression construct for alpha 3—pcDNA3-KEalpha3

FIG. 13 depicts the expression construct for alpha 5—pHook3-KEalpha5RBS

FIG. 14 depicts the expression construct for beta 2—pcDNA3-KEbeta2RBS

DETAILED DESCRIPTION OF THE  
INVENTION

In accordance with the present invention, we have isolated and characterized DNAs encoding novel human alpha and beta subunits of neuronal nAChR. Specifically, isolated DNAs encoding human  $\alpha_4$ ,  $\alpha_7$ , and  $\beta_4$  subunits of neuronal Anchors are described herein. Recombinant messenger RNA (mRNA) and recombinant polypeptides encoded by the above-described DNA are also provided.

In accordance with the present invention, we have developed methods for identifying compounds that modulate the activity of nAChRs, which employ DNAs encoding human  $\alpha$  and  $\beta$  subunits of neuronal nAChRs and polypeptides encoded thereby. Specifically, screening methods employing DNAs encoding human  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_6$ ,  $\alpha_7$ ,  $\beta_2$ ,  $\beta_3$ ,  $\beta_4$  subunits of neuronal nAChRs is described herein.

Also described are isolated cells experiencing various multimeric combinations of human  $\alpha$  and  $\beta$  subunits of neuronal nAChRs, i.e., 3-, 4- and 5-way combinations. A non-human cell line expressing human  $\alpha_7$  subunit is also described herein.

As used herein, isolated (or substantially pure) as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their in vivo cellular environments through the efforts of human beings. Thus as used herein, isolated (or substantially pure) DNA refers to DNAs purified according to standard techniques employed by those skilled in the art (see, e.g., Maniatis et al. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Similarly, as used herein, "recombinant" as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been prepared by the efforts of human beings, e.g., by cloning, recombinant expression, and the like. Thus as used herein, recombinant proteins, for example, refers to proteins produced by a recombinant host, expressing DNAs which have been added to that host through the efforts of human beings.

As used herein, a human alpha subunit gene is a gene that encodes an alpha subunit of a human neuronal nicotinic acetylcholine receptor. The alpha subunit is a subunit of the nAChR to which ACh binds. Assignment of the name "alpha" to a putative nAChR subunit, according to Deneris et al. [Tips (1991) 12:34-40] is based on the conservation of adjacent cysteine residues in the presumed extracellular domain of the subunit that are the homologues of cysteines 192 and 193 of the Torpedo alpha subunit (see Noda et al. (1982) *Nature* 299:793-797). As used herein, an alpha subunit subtype refers to a human nAChR subunit that is encoded by DNA that hybridizes under high stringency conditions to at least one of the nAChR alpha subunit-encoding DNAs (or deposited clones) disclosed herein. An alpha subunit also binds to ACh under physiological conditions and at physiological concentrations and, in the optional presence of a beta subunit (i.e., some alpha subunits are functional alone, while others require the presence of a beta subunit), generally forms a functional AChR as assessed by methods described herein or known to those of skill in this art.

Also contemplated are alpha subunits encoded by DNAs that encode alpha subunits as defined above, but that by virtue of degeneracy of the genetic code do not necessarily hybridize to the disclosed DNA or deposited clones under specified hybridization conditions. Such subunits also form a functional receptor, as assessed by the methods described

herein or known to those of skill in the art, generally with one or more beta subunit subtypes. Typically, unless an alpha subunit is encoded by RNA that arises from alternative splicing (i.e., a splice variant), alpha-encoding DNA and the alpha subunit encoded thereby share substantial sequence homology with at least one of the alpha subunit DNAs (and proteins encoded thereby) described or deposited herein. It is understood that DNA or RNA encoding a splice variant may overall share less than 90% homology with the DNA or RNA provided herein, but include regions of nearly 100% homology to a DNA fragment or deposited clone described herein, and encode an open reading frame that includes start and stop codons and encodes a functional alpha subunit.

As used herein, a splice variant refers to variant NACHR subunit-encoding nucleic acid(s) produced by differential processing of primary transcript(s) of genomic DNA, resulting in the production of more than one type of mRNA. cDNA derived from differentially processed genomic DNA will encode NACHR subunits that have regions of complete amino acid identity and regions having different amino acid sequences. Thus, the same genomic sequence can lead to the production of multiple, related mRNAs and proteins. Both the resulting mRNAs and proteins are referred to herein as "splice variants".

Stringency of hybridization is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature ( $T_m$ ) of the hybrids.  $T_m$  can be approximated by the formula:

$$81.5^\circ \text{C.} - 16.6(\log_{10} [\text{Na}^+]) + 0.41(\% \text{G+C}) - 600/1,$$

where 1 is the length of the hybrids in nucleotides.  $T_m$  decreases approximately  $1^\circ$ - $1.5^\circ$  C. with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions. Thus, as used herein:

- (1) HIGH STRINGENCY refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at  $65^\circ$  C. (i.e., if a hybrid is not stable in 0.018M NaCl at  $65^\circ$  C., it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5 $\times$  Denhardt's solution, 5 $\times$ SSPE, 0.2% SDS at  $42^\circ$  C., followed by washing in 0.1 $\times$ SSPE, and 0.1% SDS at  $65^\circ$  C.;
- (2) MODERATE STRINGENCY refers to conditions equivalent to hybridization in 50% formamide, 5 $\times$  Denhardt's solution, 5 $\times$ SSPE, 0.2% SDS at  $42^\circ$  C., followed by washing in 0.2 $\times$ SSPE, 0.2% SDS, at  $65^\circ$  C.; and
- (3) LOW STRINGENCY refers to conditions equivalent to hybridization in 10% formamide, 5 $\times$  Denhardt's solution, 6 $\times$ SSPE, 0.2% SDS, followed by washing in 1 $\times$ SSPE, 0.2% SDS, at  $50^\circ$  C.

It is understood that these conditions may be duplicated using a variety of buffers and temperatures and that they are not necessarily precise.

Denhardt's solution and SSPE (see, e.g., Sambrook, Fritsch, and Maniatis, in: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers. For example, SSPE is pH 7.4

phosphate-buffered 0.18M NaCl. SSPE can be prepared, for example, as a 20× stock solution by dissolving 175.3 g of NaCl, 27.6 g of NaH<sub>2</sub> PO<sub>4</sub> and 7.4 g EDTA in 800 ml of water, adjusting the pH to 7.4, and then adding water to 1 liter. Denhardt's solution (see, Denhardt (1966) *Biochem. Biophys. Res. Commun.* 23:641) can be prepared, for example, as a 50× stock solution by mixing 5 g Ficoll (Type 400, Pharmacia LKB Biotechnology, INC., Piscataway N.J.), 5 g of polyvinylpyrrolidone, 5 g bovine serum albumin (Fraction V; Sigma, St. Louis Mo.) water to 500 ml and filtering to remove particulate matter.

The phrase "substantial sequence homology" is used herein in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the amino acid sequence of protein, that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species having substantial sequence homology are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" mean that "homologous" sequences, i.e., sequences that have substantial homology with the DNA, RNA, or proteins disclosed and claimed herein, are functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

In practice, the term substantially the same sequence means that DNA or RNA encoding two proteins hybridize under conditions of high stringency and encode proteins that have the same sequence of amino acids or have changes in sequence that do not alter their structure or function. As used herein, substantially identical sequences of nucleotides share at least about 90% identity, and substantially identical amino acid sequences share more than 95% amino acid identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

As used herein, " $\alpha_2$  subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ. ID. No:1, or to the DNA of deposited clone having ATCC Accession No. 68277, or to DNA that encodes the amino acid sequence set forth in SEQ. ID. No:2. Typically, unless an  $\alpha_2$  subunit arises as a splice variant, an  $\alpha_2$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_2$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA described herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, " $\alpha_3$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ. ID. No:3, or to the DNA of deposited clone

having ATCC Accession No. 68278, or to DNA that encodes the amino acid sequence set forth in SEQ. ID. No:4. Typically, unless an  $\alpha_3$  arises as a splice variant, an  $\alpha_3$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_3$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above described DNA.

As used herein, " $\alpha_5$  subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, as described, for example, by Chini et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:1572-1576.

The phrase "substantially the same" is used herein in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the amino acid sequence of protein, that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species that are substantially the same are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" mean that sequences that are substantially the same as the DNA, RNA, or proteins disclosed and claimed herein are functionally equivalent to the human-derived sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the human-derived nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode human-derived proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution on a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

As used herein, " $\alpha_4$  subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example

said DNA may encode the amino acid sequence set forth in SEQ. ID. No:6, or

said DNA may encode the amino acid sequence encoded by clone HnAChR $\alpha$ 4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA may encode the amino acid sequence encoded by clone HnAChR $\alpha$ 4.1, deposited under ATCC Accession No. 69152.

Presently preferred  $\alpha_4$ -encoding DNAs can be characterized as follows

said DNA may hybridize to the coding sequence set forth in SEQ. ID. No:5 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 184-2067) under high stringency conditions, or

said DNA may hybridize under high stringency conditions to the sequence (preferably to substantially the entire sequence) of the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha$ 4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA may hybridize under high stringency conditions to the sequence of the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha$ 4.1, deposited under ATCC Accession No. 69152.

Especially preferred  $\alpha_4$ -encoding DNAs of the invention are characterized as follows

DNA having substantially the same nucleotide sequence as the coding region set forth in SEQ. ID. No:5 (i.e., nucleotides 184–2067 thereof), or

DNA having substantially the same nucleotide sequence as the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha_4.2$ , deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA have substantially the same sequence as the  $\alpha_4$ -encoding insert of clone HnAChR $\alpha_4.1$ , deposited under ATCC Accession No. 69152.

Typically, unless an  $\alpha_4$  subunit arises as a splice variant,  $\alpha_4$ -encoding DNA will share substantial sequence homology (i.e., greater than about 90%), with the  $\alpha_4$  DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNAs.

As used herein, " $\alpha_3$  subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 7, or to DNA that encodes the amino acid sequence set forth in SEQ ID No. 8. Typically, unless an  $\alpha_5$  subunit arises as a splice variant, an  $\alpha_5$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_5$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA described herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA. Human  $\alpha_5$  subunit DNA has been described in the art, for example, by Chini et al. (1992) Proc. Natl. Acad. Sci. USA 89: 1572–1576.

As used herein, " $\alpha_6$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 9, or to DNA that encodes the amino acid sequence set forth in SEQ ID No. 10. Typically, unless an  $\alpha_6$  arises as a splice variant, an  $\alpha_6$  DNA shares substantial sequence homology (greater than about 90%) with the  $\alpha_6$  DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above described DNA.

As used herein, " $\alpha_7$  subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example, the nucleotides of said DNA may encode the amino acid sequence set forth in SEQ ID No: 12. Presently preferred  $\alpha_7$ -encoding DNAs can be characterized as DNA which hybridizes under high stringency conditions to the coding sequence set forth in SEQ ID No: 11 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 73–1581). Especially preferred  $\alpha_7$ -encoding DNAs of the invention are characterized as having substantially the same nucleotide sequence as the coding sequence set forth in SEQ ID No: 11 (i.e., nucleotides 73–1581 thereof).

Typically, unless an  $\alpha_7$  subunit arises as a splice variant,  $\alpha_7$ -encoding DNA will share substantial sequence homology (greater than about 90%) with the  $\alpha_7$  DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such DNA would include regions of nearly 100% homology to the above-described DNA.

The  $\alpha_7$  subunits derived from the above-described DNA are expected to bind to the neurotoxin  $\alpha$ -bungarotoxin ( $\alpha$ -bgtx). The activity of AChRs that contain  $\alpha_7$  subunits should be inhibited upon interaction with  $\alpha$ -bgtx. Amino acid residues 210 through 217, as set forth in SEQ. ID. No:8, are believed to be important elements in the binding of  $\alpha$ -bgtx (see, for example, Chargeux et al. Trends Pharmacol Sci. (1992) 13:299–301).

As used herein, a human beta subunit gene is a gene that encodes a beta subunit of a human neuronal nicotinic acetylcholine receptor. Assignment of the name "beta" to a putative nNACHR subunit, according to Deneris et al. supra, is based on the lack of adjacent cysteine residues (which are characteristic of alpha subunits). The beta subunit is frequently referred to as the structural NACHR subunit (although it is possible that beta subunits also have ACh binding properties). Combination of beta subunit(s) with appropriate alpha subunit(s) leads to the formation of a functional receptor. As used herein, a beta subunit subtype refers to a nNACHR subunit that is encoded by DNA that hybridizes under high stringency conditions to at least one of the nNACHR-encoding DNAs (or deposited clones) disclosed herein. A beta subunit forms a functional NACHR, as assessed by methods described herein or known to those of skill in this art, with appropriate alpha subunit subtype(s).

Also contemplated are beta subunits encoded by DNAs that encode beta subunits as defined above, but that by virtue of degeneracy of the genetic code do not necessarily hybridize to the disclosed DNA or deposited clones under the specified hybridization conditions. Such subunits also form functional receptors, as assessed by the methods described herein or known to those of skill in the art, in combination with appropriate alpha subunit subtype(s). Typically, unless a beta subunit is encoded by RNA that arises as a splice variant, beta-encoding DNA and the beta subunit encoded thereby share substantial sequence homology with the beta-encoding DNA and beta subunit protein described herein. It is understood that DNA or RNA encoding a splice variant may share less than 90% overall homology with the DNA or RNA provided herein, but such DNA will include regions of nearly 100% homology to the DNA described herein.

As used herein, " $\beta_2$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 13, or to the DNA of deposited clone HnAChR $\beta_2$ , having ATCC Accession No. 68279, or to DNA encoding the amino acid sequence set forth in SEQ ID No. 14. Typically, unless a  $\beta_2$  subunit arises as a splice variant, a  $\beta_2$  DNA shares substantial sequence homology (greater than about 90%) with the  $\beta_2$  DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, " $\beta_3$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 15, or to DNA encoding the amino acid sequence set forth in SEQ ID No. 16. Typically, unless a  $\beta_3$  subunit arises as a splice variant, a  $\beta_3$  DNA shares substantial sequence homology (greater than about 90%) with the  $\beta_3$  DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, " $\beta_4$  subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the

same name. Such DNA can be characterized in a number of ways, for example, the nucleotides of said DNA may encode the amino acid sequence set forth in SEQ. ID. No:18. Presently preferred  $\beta_4$ -encoding DNAs can be characterized as DNA which hybridizes under high stringency conditions to the coding sequence set forth in SEQ. ID. No:17 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 87–1583). Especially preferred  $\beta_4$ -encoding DNAs of the invention are characterized as having substantially the same nucleotide sequence as set forth in SEQ. ID. No:17.

Typically, unless a  $\beta_4$  subunit arises as a splice variant,  $\beta_4$ -encoding DNA will share substantial sequence homology (greater than about 90%) with the  $\beta_4$  DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such DNA would include regions of nearly 100% homology to the above-described DNA.

DNA encoding human neuronal nicotinic AChR alpha and beta subunits may be isolated by screening suitable human cDNA or human genomic libraries under suitable hybridization conditions with DNA disclosed herein (including nucleotides derived from any of SEQ. ID. Nos:1, 3, 5, 7, 9, 11, 13, 15 or 17, or with any of the deposited clones referred to herein. Suitable libraries can be prepared from neuronal tissue samples, hippocampus tissue, or cell lines, such as the human neuroblastoma cell line IMR32 (ATCC Accession No. CCL127), and the like. The library is preferably screened with a portion of DNA including the entire subunit-encoding sequence thereof, or the library may be screened with a suitable probe.

As used herein, a probe is single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or the complement of) any 14 bases set forth in any of SEQ. ID. Nos:1, 3, 5, 7, 9, or 11, or in the subunit encoding DNA in any of the deposited clones described herein (e.g., ATCC accession no. 69239 or 69152). Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode transmembrane domains, sequences predicted to encode the cytoplasmic loop, signal sequences, acetylcholine (ACh) and  $\alpha$ -bungarotoxin ( $\alpha$ -bgtx) binding sites, and the like. Amino acids 210–220 are typically involved in ACh and  $\alpha$ -bgtx binding. The approximate amino acid residues which comprise such regions for other preferred probes are set forth in the following table:

Sub-unit	Signal Sequence	TMD1*	TMD2	TMD3	TMD4	Cytoplasmic Loop
$\alpha_2$	1–55	264–289	297–320	326–350	444–515	351–443
$\alpha_3$	1–30	240–265	273–296	302–326	459–480	327–458
$\alpha_4$	1–33	241–269	275–289	303–330	593–618	594–617
$\alpha_5$	1–22	250–275	282–304	310–335	422–437	336–421
$\alpha_6$	1–30	240–265	272–294	301–326	458–483	327–457
$\alpha_7$	1–23	229–256	262–284	290–317	462–487	318–461
$\beta_2$	1–25	234–259	267–288	295–320	453–477	321–452
$\beta_3$	1–20	232–258	265–287	293–318	421–446	319–420

-continued

Sub-unit	Signal Sequence	TMD1*	TMD2	TMD3	TMD4	Cytoplasmic Loop
$\beta_4$	1–23	234–258	264–285	290–319	454–478	320–453

\*TMD = transmembrane domain

Alternatively, portions of the DNA can be used as primers to amplify selected fragments in a particular library.

After screening the library, positive clones are identified by detecting a hybridization signal; the identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, by comparison with the sequences set forth herein or with the deposited clones described herein, to ascertain whether they include DNA encoding a complete alpha or beta subunit. If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If desired, the library can be rescreened with positive clones until overlapping clones that encode an entire alpha or beta subunit are obtained. If the library is a cDNA library, then the overlapping clones will include an open reading frame. If the library is genomic, then the overlapping clones may include exons and introns. In both instances, complete clones may be identified by comparison with the DNA and encoded proteins provided herein.

Complementary DNA clones encoding various subtypes of human nNACHR alpha and beta subunits have been isolated. Each subtype of the subunit appears to be encoded by a different gene. The DNA clones provided herein may be used to isolate genomic clones encoding each subtype and to isolate any splice variants by screening libraries prepared from different neural tissues. Nucleic acid amplification techniques, which are well known in the art, can be used to locate splice variants of human NACHR subunits. This is accomplished by employing oligonucleotides based on DNA sequences surrounding divergent sequence(s) as primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal the existence of splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns that correspond to different splice variants of transcripts encoding human NACHR subunits.

It has been found that not all subunit subtypes are expressed in all neural tissues or in all portions of the brain. Thus, in order to isolate cDNA encoding particular subunit subtypes or splice variants of such subtypes, it is preferable to screen libraries prepared from different neuronal or neural tissues. Preferred libraries for obtaining DNA encoding each subunit include: hippocampus to isolate human  $\alpha_4$ - and  $\alpha_5$ -encoding DNA; IMR32 to isolate human  $\alpha_3$ -,  $\alpha_5$ -,  $\alpha_7$ - and  $\beta_4$ -encoding DNA, thalamus to isolate  $\alpha_2$  and  $\beta$ -encoding DNA; and the like.

It appears that the distribution of expression of human neuronal nicotinic AChRs differs from the distribution of such receptors in rat. For example, RNA encoding the rat  $\alpha_4$  subunit is abundant in rat thalamus, but is not abundant in rat hippocampus (see, e.g., Wada et al. (1989) J. Comp. Neurol 284:314–335). No  $\alpha_4$ -encoding clones could be obtained, however, from a human thalamus library. Instead, human  $\alpha_4$  clones were ultimately obtained from a human hippocampus library. Thus, the distribution of  $\alpha_4$  nNACHR subunit in humans and rats appears to be quite different.

Rat  $\alpha_3$  subunit appears to be a CNS-associated subunit that is abundantly expressed in the thalamus and weakly

expressed in the brain stem (see, e.g., Boulter et al. (1986) *Nature* 319:368–374; Boulter et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7763–7767; and Wada et al. (1989) *J. Comp. Neurol* 284:314–335). In efforts to clone DNA encoding the human nicotinic AChR  $\alpha_3$  subunit, however, several human libraries, including a thalamus library, were unsuccessfully screened. Surprisingly, clones encoding human  $\alpha_3$  subunit were ultimately obtained from a brain stem library and from IMR32 cells that reportedly express few, if any, functional nicotinic acetylcholine receptors (see, e.g., Gotti et al. (1986) *Biochem. Biophys. Res. Commun.* 137:1141–1147, and Clementi et al. (1986) *J. Neurochem.* 47:291–297).

Rat  $\alpha_7$  subunit transcript reportedly is abundantly expressed in the hippocampus (see Seguela et al. (1993) *J. Neurosci.* 13:596–604). Efforts to clone DNA encoding a human  $\alpha_7$  subunit from a human hippocampus library ( $1 \times 10^6$  recombinants) were unsuccessful. Surprisingly, clones encoding a human NACHR  $\alpha_7$  subunit were ultimately obtained from an IMR32 cell cDNA library.

The above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the level of skill of the art.

An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of affecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Presently preferred plasmids for expression of invention AChR subunits in eukaryotic host cells, particularly mammalian cells, include SV40 promoter-based expression vectors, such as pZeoSV (Invitrogen, San Diego, Calif.) CMV; cytomegalovirus (CMV) promoter-based vectors such as, pcDNA1, pcDNA3, pCEP4, (Invitrogen, San Diego, Calif.); and MMTV promoter-based vector such as pMAMneo (Clontech, Inc.) and the like.

As used herein, a promoter region refers to a segment of DNA that controls transcription of DNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors.

Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the like.

As used herein, the term “operatively linked” refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a

promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to remove or alter 5' untranslated portions of the clones to remove extra, potential alternative translation initiation (i.e., start) codons or other sequences that interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, for example, Kozak (1991) *J. Biol. Chem.* 266:19867–19870) can be inserted immediately 5' of the start codon to enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, expression refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCDNA1 (Invitrogen, San Diego, Calif.), and MMTV promoter-based vectors such as pMSG (Catalog No. 27-4506-01 from Pharmacia, Piscataway, N.J.).

Full-length DNAs encoding human neuronal NACHR subunits have been inserted into vector pCMV-T7, a pUC19-based mammalian cell expression vector containing the CMV promoter/enhancer, SV40 splice/donor sites located immediately downstream of the promoter, a polylinker downstream of the splice/donor sites, followed by an SV40 polyadenylation signal. Placement of NACHR subunit DNA between the CMV promoter and SV40 polyadenylation signal provides for constitutive expression of the foreign DNA in a mammalian host cell transfected with the construct. For inducible expression of human NACHR subunit-encoding DNA in a mammalian cell, the DNA can be inserted into a plasmid such as PMSG. This plasmid contains the mouse mammary tumor virus (MMTV) promoter for steroid-inducible expression of operatively associated foreign DNA. If the host cell does not express endogenous glucocorticoid receptors required for uptake of glucocorticoids (i.e., inducers of the MMTV promoter) into the cell, it is necessary to additionally transfect the cell with DNA encoding the glucocorticoid receptor (ATCC accession no. 67200). Full-length human DNA clones encoding human  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_7$ ,  $\beta_2$  and  $\beta_4$  have also been subcloned into pIBI24 (International Biotechnologies, Inc., New Haven, Conn.) or pCMV-T7-2 for synthesis of in vitro transcripts.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described polynucleic acids (i.e., DNA or mRNA). Such host cells as bacterial, yeast and mammalian cells can be used for replicating DNA and producing nAChR subunit(s). Methods for constructing expression vectors, preparing in vitro transcripts, transfecting DNA into mammalian cells, injecting oocytes, and performing electrophysiological and other analyses for assessing receptor expression and function as described herein are also described in PCT Application Nos. PCT/US91/02311, PCT/US91/05625 and PCT/US92/11090, and in co-pending U.S. application Ser. Nos. 07/504,455, 07/563,751 and 07/812,254. The subject matter



of these applications are hereby incorporated by reference herein in their entirety.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press). Heterologous DNA may be introduced into host cells by any method known to those of skill in the art, such as transfection with a vector encoding the heterologous DNA by CaPO<sub>4</sub> precipitation (see, e.g., Wigler et al. (1979) Proc. Natl. Acad. Sci. 76:1373–1376). Recombinant cells can then be cultured under conditions whereby the subunit(s) encoded by the DNA is (are) expressed. Preferred cells include mammalian cells (e.g., HEK 293, CHO and Ltk<sup>-</sup> cells), yeast cells (e.g., methylotrophic yeast cells, such as *Pichia pastoris*), bacterial cells (e.g., *Escherichia coli*), and the like.

While the DNA provided herein may be expressed in any eukaryotic cell, including yeast cells (such as, for example, *P. pastoris* (see U.S. Pat. Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), *Saccharomyces cerevisiae*, *Candida tropicalis*, *Hansenula polymorpha*, and the like), mammalian expression systems, including commercially available systems and other such systems known to those of skill in the art, for expression of DNA encoding the human neuronal nicotinic AChR subunits provided herein are presently preferred. *Xenopus* oocytes are preferred for expression of RNA transcripts of the DNA.

In preferred embodiments, DNA is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express a specific human nAChR receptor subtype, or specific combinations of subtypes. The resulting cell lines can then be produced in quantity for reproducible quantitative analysis of the effects of drugs on receptor function. In other embodiments, mRNA may be produced by in vitro transcription of DNA encoding each subunit. This mRNA, either from a single subunit clone or from a combination of clones, can then be injected into *Xenopus* oocytes where the RNA directs the synthesis of the human receptor subunits, which then form functional receptors. Alternatively, the subunit-encoding DNA can be directly injected into oocytes for expression of functional receptors. The transfected mammalian cells or injected oocytes may then be used in the methods of drug screening provided herein.

Cloned full-length DNA encoding any of the subunits of human neuronal nicotinic AChR may be introduced into a plasmid vector for expression in a eukaryotic cell. Such DNA may be genomic DNA or cDNA. Host cells may be transfected with one or a combination of plasmids, each of which encodes at least one human neuronal nicotinic AChR subunit.

Eukaryotic cells in which DNA or RNA may be introduced include any cells that are transfectable by such DNA or RNA or into which such DNA or RNA may be injected. Preferred cells are those that can be transiently or stably transfected and also express the DNA and RNA. Presently most preferred cells are those that can form recombinant or heterologous human neuronal nicotinic AChRs comprising one or more subunits encoded by the heterologous DNA. Such cells may be identified empirically or selected from among those known to be readily transfected or injected.

Exemplary cells for introducing DNA include cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese

hamster ovary (CHO) cells, human embryonic kidney cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g., *Xenopus laevis* oocytes), yeast cells (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK 293 (which are available from ATCC under accession #CRL 1573; Ltk<sup>-</sup> cells (which are available from ATCC under accession #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhfr<sup>-</sup> CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12:555). Presently preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that have been adapted for growth in suspension and that can be frozen in liquid nitrogen and then thawed and regrown. HEK 293 cells are described, for example, in U.S. Pat. No. 5,024,939 to Gorman (see, also, Stillman et al. (1985) Mol. Cell. Biol. 5:2051–2060).

DNA may be stably incorporated into cells or may be transiently introduced using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene (such as, for example, the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance, and the like), and growing the transfected cells under conditions selective for cells expressing the marker gene. To produce such cells, the cells should be transfected with a sufficient concentration of subunit-encoding nucleic acids to form human neuronal nicotinic AChRs that contain the human subunits encoded by heterologous DNA. The precise amounts and ratios of DNA encoding the subunits may be empirically determined and optimized for a particular combination of subunits, cells and assay conditions. Recombinant cells that express neuronal nicotinic AChR containing subunits encoded only by the heterologous DNA or RNA are especially preferred.

Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing recombinant cells are known to the skilled artisan. Similarly, the human neuronal nicotinic AChR subunits may be purified using protein purification methods known to those of skill in the art. For example, antibodies or other ligands that specifically bind to one or more of the subunits may be used for affinity purification of the subunit or human neuronal nicotinic AChRs containing the subunits.

In accordance with one embodiment of the present invention, methods for producing cells that express human neuronal nicotinic AChR subunits and functional receptors are also provided. In one such method, host cells are transfected with DNA encoding at least one alpha subunit of a neuronal nicotinic acetylcholine receptor and at least one beta subunit of a neuronal nicotinic acetylcholine receptor. Using methods such as northern blot or slot blot analysis, transfected cells that contain alpha and/or beta subunit encoding DNA or RNA can be selected. Transfected cells are also analyzed to identify those that express nAChR protein. Analysis can be carried out, for example, by measuring the ability of cells to bind acetylcholine, nicotine, or a nicotine agonist, compared to the nicotine binding ability of untransfected host cells or other suitable control cells, by electrophysiologically monitoring the currents through the cell membrane in response to a nicotine agonist, and the like.

In particularly preferred aspects, eukaryotic cells which contain heterologous DNAs express such DNA and form recombinant functional neuronal nicotinic AChR(s). In more preferred aspects, recombinant neuronal nicotinic AChR activity is readily detectable because it is a type that is absent from the untransfected host cell or is of a magnitude not exhibited in the untransfected cell. Such cells that contain recombinant receptors could be prepared, for example, by causing cells transformed with DNA encoding the human neuronal nicotinic AChR  $\alpha_3$  and  $\beta_4$  subunits to express the corresponding proteins. The resulting synthetic or recombinant receptor would contain only the  $\alpha_3$  and  $\beta_4$  nNACHR subunits. Such a receptor would be useful for a variety of applications, e.g., as part of an assay system free of the interferences frequently present in prior art assay systems employing non-human receptors or human tissue preparations. Furthermore, testing of single receptor subunits with a variety of potential agonists or antagonists would provide additional information with respect to the function and activity of the individual subunits. Such information may lead to the identification of compounds which are capable of very specific interaction with one or more of the receptor subunits. Such specificity may prove of great value in medical application.

Thus, DNA encoding one or more human neuronal nicotinic AChR subunits may be introduced into suitable host cells (e.g., eukaryotic or prokaryotic cells) for expression of individual subunits and functional NACHRs. Preferably combinations of alpha and beta subunits may be introduced into cells: such combinations include combinations of any one or more of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$  and  $\alpha_7$  with  $\beta_2$  or  $\beta_4$ . Sequence information for  $\alpha_1$  is presented in Biochem. Soc. Trans. (1989) 17:219-220; sequence information for  $\alpha_5$  is presented in Proc. Natl. Acad. Sci. USA (1992) 89:1572-1576; and sequence information for  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_7$ ,  $\beta_2$  and  $\beta_4$  is presented in the Sequence Listing provided herewith. Presently preferred combinations of subunits include any one or more of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  with  $\beta_4$ ; or  $\alpha_4$  or  $\alpha_7$  in combination with either  $\beta_2$  or  $\beta_4$ . It is recognized that some of the subunits may have ion transport function in the absence of additional subunits. For example, the  $\alpha_7$  subunit is functional in the absence of any added beta subunit.

In accordance with the above, also disclosed are cells transfected or transformed with DNA or RNA encoding multimeric human NACHR subunit combinations. These include but are not limited to the following:

#### Multimeric Subunit Combinations

$\alpha_2\beta_4\alpha_6$   
 $\alpha_3\beta_4\alpha_6$   
 $\alpha_4\beta_4\alpha_5$   
 $\alpha_4\beta_4\alpha_6$   
 $\alpha_4\beta_2\alpha_5$   
 $\alpha_4\beta_2\beta_3$   
 $\alpha_3\beta_2\alpha_6\beta_3$   
 $\alpha_2\beta_4\alpha_5$   
 $\alpha_2\beta_2\alpha_5$   
 $\alpha_3\beta_2\alpha_5$   
 $\alpha_3\beta_4\alpha_5$

Also contemplated are cells expressing one or more  $\alpha$  subunit with more than one  $\alpha$  subunit. These include but are not limited to the following subunit combinations:

$\alpha X\beta_2\beta_4$  (where X defines one or more of the alpha subunits disclosed herein)  
 $\alpha X\beta_2\beta_3\beta_4$   
 $\alpha_2\beta_2\alpha_6$

$\alpha_3\beta_2\alpha_6$

$\alpha_4\beta_2\alpha_6$

$\alpha X\beta_2\beta_3$  (where X defines one or more of the alpha subunits disclosed herein)

Stable cell lines expressing any of the above referenced multimeric subunit combinations are also a feature of the invention.

As used herein, " $\beta_2$  subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ. ID. No:9, or to the DNA of deposited clone HnACh $\beta_62$ , having ATCC Accession No. 68279, or to DNA encoding the amino acid sequence set forth in SEQ. ID. No:10. Typically, unless a  $\beta_2$  subunit arises as a splice variant, a  $\beta_2$  DNA shares substantial sequence homology (greater than about 90%) with the  $\beta_2$  DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

In certain embodiments, eukaryotic cells with heterologous human neuronal nicotinic AChRs are produced by introducing into the cell a first composition, which contains at least one RNA transcript that is translated in the cell into a subunit of a human neuronal nicotinic AChR. In preferred embodiments, the subunits that are translated include an alpha subunit of a human neuronal nicotinic AChR. More preferably, the composition that is introduced contains an RNA transcript which encodes an alpha subunit and also contains an RNA transcript which encodes a beta subunit of a human neuronal nicotinic AChR. RNA transcripts can be obtained from cells transfected with DNAs encoding human neuronal nicotinic acetylcholine receptor subunits or by in vitro transcription of subunit-encoding DNAs. Methods for in vitro transcription of cloned DNA and injection of the resulting mRNA into eukaryotic cells are well known in the art.

Amphibian oocytes are particularly preferred for expression of in vitro transcripts of the human nNACHR DNA clones provided herein. See, for example, Dascal (1989) CRC Crit. Rev. Biochem. 22:317-387, for a review of the use of *Xenopus* oocytes to study ion channels.

Thus, pairwise (or stepwise) introduction of DNA or RNA encoding alpha and beta subtypes into cells is possible. The resulting cells may be tested by the methods provided herein or known to those of skill in the art to detect functional AChR activity. Such testing will allow the identification of pairs of alpha and beta subunit subtypes that produce functional AChRs, as well as individual subunits that produce functional AChRs.

An alternative embodiment is drawn to a non-human cell line that stably expresses the  $\alpha_7$  nAChR. Preferably, the non-human cell line expressing the human  $\alpha_7$  nAChR subunit is a rat cell line, i.e., the GH $_4$ C $_1$  cell line.

As used herein, GH $_4$ C $_1$  cells are derived from rat pituitary tumor tissue and are transfected with DNA or RNA encoding the human  $\alpha_7$  nAChR.

As used herein, activity of a human neuronal nicotinic AChR refers to any activity characteristic of an NACHR. Such activity can typically be measured by one or more in vitro methods, and frequently corresponds to an in vivo activity of a human neuronal nicotinic AChR. Such activity may be measured by any method known to those of skill in the art, such as, for example, measuring the amount of current which flows through the recombinant channel in response to a stimulus.

Methods to determine the presence and/or activity of human neuronal nicotinic AChRs include assays that mea-

sure nicotine binding,  $^{86}\text{Rb}$  ion-flux,  $\text{Ca}^{2+}$  influx, the electrophysiological response of cells, the electrophysiological response of oocytes transfected with RNA from the cells, and the like. In particular, methods are provided herein for the measurement or detection of an AChR-mediated response upon contact of cells containing the DNA or mRNA with a test compound.

As used herein, a recombinant or heterologous human neuronal nicotinic AChR refers to a receptor that contains one or more subunits encoded by heterologous DNA that has been introduced into and expressed in cells capable of expressing receptor protein. A recombinant human neuronal nicotinic AChR may also include subunits that are produced by DNA endogenous to the host cell. In certain embodiments, recombinant or heterologous human neuronal nicotinic AChR may contain only subunits that are encoded by heterologous DNA.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome of the cell in which it is present or to DNA or RNA which is found in a location or locations in the genome that differ from that in which it occurs in nature. Typically, heterologous or foreign DNA and RNA refers to DNA or RNA that is not endogenous to the host cell and has been artificially introduced into the cell. Examples of heterologous DNA include DNA that encodes a human neuronal nicotinic AChR subunit, DNA that encodes RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes, and the like. The cell that expresses heterologous DNA may contain DNA encoding the same or different expression products. Heterologous DNA need not be expressed and may be integrated into the host cell genome or maintained episomally.

Recombinant receptors on recombinant eukaryotic cell surfaces may contain one or more subunits encoded by the DNA or mRNA encoding human neuronal nicotinic AChR subunits, or may contain a mixture of subunits encoded by the host cell and subunits encoded by heterologous DNA or mRNA. Recombinant receptors may be homogeneous or may be a mixture of subtypes. Mixtures of DNA or mRNA encoding receptors from various species, such as rats and humans, may also be introduced into the cells. Thus, a cell can be prepared that expresses recombinant receptors containing only  $\alpha_3$  and  $\beta_4$  subunits, or any other combination of alpha and beta subunits provided herein. For example,  $\alpha_4$  and/or  $\alpha_7$  subunits of the present invention can be co-expressed with  $\beta_2$  and/or  $\beta_4$  receptor subunits; similarly,  $\beta_4$  subunits according to the present invention can be co-expressed with  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$  and/or  $\alpha_7$  receptor subunits. As noted previously, some of the nNACHR subunits may be capable of forming functional receptors in the absence of other subunits, thus co-expression is not always required to produce functional receptors.

As used herein, a functional neuronal nicotinic AChR is a receptor that exhibits an activity of neuronal nicotinic AChRs as assessed by any in vitro or in vivo assay disclosed herein or known to those of skill in the art. Possession of any such activity that may be assessed by any method known to those of skill in the art and provided herein is sufficient to designate a receptor as functional. Methods for detecting NACHR protein and/or activity include, for example, assays that measure nicotine binding,  $^{86}\text{Rb}$  ion-flux,  $\text{Ca}^{2+}$  influx, the electrophysiological response of cells containing heterologous DNA or mRNA encoding one or more receptor subunit subtypes, and the like. Since all combinations of alpha and beta subunits may not form functional receptors,

numerous combinations of alpha and beta subunits should be tested in order to fully characterize a particular subunit and cells which produce same. Thus, as used herein, "functional" with respect to a recombinant or heterologous human neuronal nicotinic AChR means that the receptor channel is able to provide for and regulate entry of human neuronal nicotinic AChR-permeable ions, such as, for example,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ , in response to a stimulus and/or bind ligands with affinity for the receptor. Preferably such human neuronal nicotinic AChR activity is distinguishable, such as by electrophysiological, pharmacological and other means known to those of skill in the art, from any endogenous nicotinic AChR activity that may be produced by the host cell.

In accordance with a particular embodiment of the present invention, recombinant human neuronal nicotinic AChR-expressing mammalian cells or oocytes can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the AChR-mediated response in the presence and absence of test compound, or by comparing the AChR-mediated response of test cells, or control cells (i.e., cells that do not express nNACHRs), to the presence of the compound.

As used herein, a compound or signal that "modulates the activity of a neuronal nicotinic AChR" refers to a compound or signal that alters the activity of NACHR so that activity of the NACHR is different in the presence of the compound or signal than in the absence of the compound or signal. In particular, such compounds or signals include agonists and antagonists. The term agonist refers to a substance or signal, such as ACh, that activates receptor function; and the term antagonist refers to a substance that interferes with receptor function. Typically, the effect of an antagonist is observed as a blocking of activation by an agonist. Antagonists include competitive and non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site specific for the agonist (e.g., ligand or neurotransmitter) for the same or closely situated site. A non-competitive antagonist or blocker inactivates the functioning of the receptor by interacting with a site other than the site that interacts with the agonist.

As understood by those of skill in the art, assay methods for identifying compounds that modulate human neuronal nicotinic AChR activity (e.g., agonists and antagonists) generally require comparison to a control. One type of a "control" cell or "control" culture is a cell or culture that is treated substantially the same as the cell or culture exposed to the test compound, except the control culture is not exposed to test compound. For example, in methods that use voltage clamp electrophysiological procedures, the same cell can be tested in the presence and absence of test compound, by merely changing the external solution bathing the cell. Another type of "control" cell or "control" culture may be a cell or a culture of cells which are identical to the transfected cells, except the cells employed for the control culture do not express functional human neuronal nicotinic AChRs. In this situation, the response of test cell to test compound is compared to the response (or lack of response) of receptor-negative (control) cell to test compound, when cells or cultures of each type of cell are exposed to substantially the same reaction conditions in the presence of compound being assayed.

The functional recombinant human neuronal nicotinic AChR includes at least an alpha subunit, or an alpha subunit and a beta subunit of a human neuronal nicotinic AChR. Eukaryotic cells expressing these subunits have been prepared by injection of RNA transcripts and by transfection of

DNA. Such cells have exhibited nicotinic AChR activity attributable to human neuronal nicotinic AChRs that contain one or more of the heterologous human neuronal nicotinic AChR subunits. For example, *Xenopus laevis* oocytes that had been injected with in vitro transcripts of the DNA encoding human neuronal nicotinic AChR  $\alpha_3$  and  $\beta_4$  subunits exhibited AChR agonist induced currents; whereas cells that had been injected with transcripts of either the  $\alpha_3$  or  $\beta_4$  subunit alone did not. In addition, HEK 293 cells that had been co-transfected with DNA encoding human neuronal NACHR  $\alpha_3$  and  $\beta_4$  subunits exhibited AChR agonist-induced increases in intracellular calcium concentration, whereas control HEK 293 cells (i.e., cells that had not been transfected with  $\alpha_3$ - and  $\beta_4$ -encoding DNA) did not exhibit any AChR agonist-induced increases in intracellular calcium concentration.

With respect to measurement of the activity of functional heterologous human neuronal nicotinic AChRs, endogenous AChR activity and, if desired, activity of AChRs that contain a mixture of endogenous host cell subunits and heterologous subunits, should, if possible, be inhibited to a significant extent by chemical, pharmacological and electrophysiological means.

#### Deposits

The deposited clones have been deposited at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md., U.S.A. 20852, under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted. In particular, upon issuance of a U.S. Patent based on this or any application claiming priority to or incorporating this application by reference thereto, all restrictions upon availability of the deposited material will be irrevocably removed.

The invention will now be described in greater detail with reference to the following non-limiting examples.

### EXAMPLE 1

#### Isolation of DNA Encoding Human nAChR Subunits

##### A. DNA Encoding a Human nAChR $\beta_4$ Subunit

Random primers were used in synthesizing cDNA from RNA isolated from the IMR32 human neuroblastoma cell line (the cells had been treated with dibutyryl cAMP and bromodeoxyuridine prior to constructing the library). The library constructed from the cDNAs was screened with a fragment of a rat nicotinic AChR  $\beta_4$  subunit cDNA. Hybridization was performed at 42° C. in 5×SSPE, 5× Denhardt's solution, 50% formamide, 200  $\mu$ g/ml herring sperm DNA and 0.2% SDS. Washes were performed in 0.1×SSPE, 0.2% SDS at 65° C. Five clones were identified that hybridized to the probe.

The five clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. The insert DNA of one of the five clones contained the complete coding sequence of a  $\beta_4$  subunit of a human nicotinic AChR (see nucleotides 87–1583 of SEQ. ID. No:11). The amino acid sequence deduced from the nucle-

otide sequence of the full-length clone has ~81% identity with the amino acid sequence deduced from the rat nicotinic AChR  $\beta_4$  subunit DNA. Several regions of the deduced rat and human  $\beta_4$  amino acid sequences are notably dissimilar: amino acids 1–23 (the human sequence has only ~36% identity with respect to the rat sequence), 352–416 (the human sequence has only ~48% identity with respect to the rat sequence), and 417–492 (the human sequence has only ~78% identity with respect to the rat sequence). Furthermore, amino acids 376–379 in the rat  $\beta_4$  subunit are not contained in the human  $\beta_4$  subunit.

##### B. DNA Encoding a Human nAChR $\alpha_7$ Subunit

An amplified IMR32 cell cDNA library ( $1 \times 10^6$  recombinants; cells treated with dibutyryl cAMP and bromodeoxyuridine) was screened with a fragment of a rat nicotinic AChR  $\alpha_7$  subunit cDNA. The hybridization conditions were identical to those described above for screening an IMR32 cell cDNA library with the rat  $\beta_4$  subunit DNA. Washes were performed in 0.2×SSPE, 0.2% SDS at 65° C. Seven positive clones were identified by hybridization to the labeled rat DNA probe. Six of the clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. One of the clones contains the complete coding sequence of a human AChR receptor  $\alpha_7$  subunit gene (see nucleotides 73–1581 of SEQ. ID. No:7).

##### C. DNA Encoding a Human nAChR $\alpha_4$ Subunit

Random primers were used in synthesizing cDNA from RNA isolated from human hippocampus tissue. cDNAs larger than 2.0 kb were inserted into the  $\lambda$ gt10 phage vector to create a cDNA library. Approximately  $1 \times 10^6$  recombinants were screened with a fragment of a DNA encoding a rat nicotinic AChR  $\alpha_4$  subunit using the same hybridization and washing conditions as described above for screening an IMR32 cell cDNA library for  $\alpha_7$  subunit cDNAs. Three clones hybridized strongly to the probe. Two of these three clones, designated KE $\alpha$ 4.1 and KE $\alpha$ 4.2, have been deposited with the American Type Culture Collection (ATCC, Rockville, Md.) and assigned accession nos. 69152 and 69239, respectively.

Characterization of the plaque-purified clones revealed that one of the clones, KE $\alpha$ 4.2, contains the complete coding sequence of a human nicotinic AChR  $\alpha_4$  subunit gene (coding sequence of this human  $\alpha_4$  subunit cDNA is provided as nucleotides 184–2067 in SEQ. ID. No:5). Comparison of the 5' ends of the coding sequences of the human and rat  $\alpha_4$  subunit cDNAs reveals that the rat sequence contains an 18-nucleotide segment that is not present in the human sequence.

##### D. DNA Encoding Human nAChR $\alpha_2$ , $\alpha_3$ , & $\beta_2$ Subunits

Plasmids containing DNA that encodes and/or that can be used to isolate DNA that encodes human neuronal nicotinic acetylcholine receptor  $\alpha_2$ ,  $\alpha_3$  and  $\beta_2$  subunits have been deposited with the American Type Culture Collection (ATCC). The clone names and deposit accession numbers are:

Subunit	Clone Name	ATCC Accession No.
$\alpha_2$	HnAChR $\alpha_2$	68277
$\alpha_3$	HnAChR $\alpha_3$	68278
$\beta_2$	HnAChR $\beta_2$	68279

In addition, DNA sequences that encode full-length  $\alpha_2$ ,  $\alpha_3$  and  $\beta_2$  subunits are set forth in SEQ. ID. Nos:1, 3 and 9, respectively.

## EXAMPLE 2

## I. Preparation of Constructs for the Expression of Recombinant Human Neuronal Nicotinic AChR Subunits

Isolated cDNAs encoding human neuronal nicotinic AChR subunits were incorporated into vectors for use in expressing the subunits in mammalian host cells and for use in generating in vitro transcripts of the DNAs to be expressed in *Xenopus* oocytes. Several different vectors were utilized in preparing the constructs as follows.

A. Construct for Expression of a Human nNACHR  $\alpha_3$  Subunit

DNA encoding a human neuronal nicotinic AChR  $\alpha_3$  subunit was subcloned into the pCMV-T7-2 general expression vector to create pCMV-KE $\alpha_3$ . Plasmid pCMV-T7-2 (see FIG. 1) is a pUC19-based vector that contains a CMV promoter/enhancer, SV40 splice donor/splice acceptor sites located immediately downstream of the promoter, a T7 bacteriophage RNA polymerase promoter positioned downstream of the SV40 splice sites, an SV40 polyadenylation signal downstream of the T7 promoter, and a polylinker between the T7 promoter and the polyadenylation signal. This vector thus contains all the regulatory elements required for expression of heterologous DNA in a mammalian host cell, wherein the heterologous DNA has been incorporated into the vector at the polylinker. In addition, because the T7 promoter is located just upstream of the polylinker, this plasmid can be used for synthesis of in vitro transcripts of heterologous DNA that has been subcloned into the vector at the polylinker. FIG. 1 also shows a restriction map of pCMV-T7-3. This plasmid is identical to pCMV-T7-2 except that the restriction sites in the polylinker are in the opposite order as compared to the order in which they occur in pCMV-T7-2.

A 1.7 kb SfiI (blunt-ended)/EcoRI DNA fragment containing nucleotides 27–1759 of SEQ. ID. No:3 (i.e., the entire  $\alpha_3$  subunit coding sequence plus 12 nucleotides of 5' untranslated sequence and 206 nucleotides of 3' untranslated sequence) was ligated to EcoRV/EcoRI-digested pCMV-T7-2 to generate pCMV-KE $\alpha_3$ . Plasmid pCMV-KE $\alpha_3$  was used for expression of the  $\alpha_3$  subunit in mammalian cells and for generating in vitro transcripts of the  $\alpha_3$  subunit DNA.

B. Constructs for Expression of a Human nNACHR  $\beta_4$  Subunit

A 1.9 kb EcoRI DNA fragment containing nucleotides 1–1915 of SEQ. ID. No:11 (i.e., the entire  $\beta_4$  subunit coding sequence plus 86 nucleotides of 5' untranslated sequence and 332 nucleotides of 3' untranslated sequence) was ligated to EcoRI-digested pGEM7Zf(+) (Promega Catalog #P2251; Madison, Wis.). The resulting construct, KE $\beta_4$ .6/pGEM, contains the T7 bacteriophage RNA polymerase promoter in operative association with two tandem  $\beta_4$  subunit DNA inserts (in the same orientation) and was used in generating in vitro transcripts of the DNA.

The same 1.9 kb EcoRI DNA fragment containing nucleotides 1–1915 of SEQ. ID. No:11 was ligated as a single insert to EcoRI-digested pCMV-T7-3 to generate pCMV-KE $\beta_4$ . Plasmid pCMV-KE $\beta_4$  was used for expression of the  $\beta_4$  subunit in mammalian cells and for generating in vitro transcripts of the  $\beta_4$  subunit DNA.

C. Constructs for Expression of a Human nNACHR  $\alpha_7$  Subunit

Two pCMV-T7-2-based constructs were prepared for use in recombinant expression of a human neuronal nicotinic AChR  $\alpha_7$  subunit. The first construct, pCMV-KE $\alpha_7$ .3, was prepared by ligating a 1.9 kb XhoI DNA fragment containing nucleotides 1–1876 of SEQ. ID. No:7 (i.e., the entire  $\alpha_7$

subunit coding sequence plus 72 nucleotides of 5' untranslated sequence and 295 nucleotides of 3' untranslated sequence) to SallI-digested pCMV-T7-3. The second construct, pCMV-KE $\alpha_7$ , was prepared by replacing the 5' untranslated sequence of the 1.9 kb XhoI  $\alpha_7$  subunit DNA fragment described above with a consensus ribosome binding site (5'-GCCACC-3'; see Kozak (1987) Nucl. Acids Res. 15:8125–8148). The resulting modified fragment was ligated as a 1.8 kb BglIII/XhoI fragment with BglIII/SallI-digested pCMV-T7-2 to generate pCMV-KE $\alpha_7$ . Thus, in pCMV-KE $\alpha_7$ , the translation initiation codon of the coding sequence of the  $\alpha_7$  subunit cDNA is preceded immediately by a consensus ribosome binding site.

D. Constructs for Expression of a Human nNACHR  $\beta_2$  Subunit

DNA fragments encoding portions of a human neuronal nicotinic AChR  $\beta_2$  subunit were ligated together to generate a full-length  $\beta_2$  subunit coding sequence contained in plasmid pIBI124 (International Biotechnologies, Inc. (IBI), New Haven, Conn.). The resulting construct, H $\beta_2$ .1F, contains nucleotides 1–2450 of SEQ. ID. No:9 (i.e., the entire  $\beta_2$  subunit coding sequence, plus 266 nucleotides of 5' untranslated sequence and 675 nucleotides of 3' untranslated sequence) in operative association with the T7 promoter. Therefore, H $\beta_2$ .1F was used for synthesis of in vitro transcripts from the  $\beta_2$  subunit DNA.

Since the 5' untranslated sequence of the  $\beta_2$  subunit DNA contains a potential alternative translation initiation codon (ATG) beginning 11 nucleotides upstream (nucleotides 256–258 in SEQ. ID. No:9) of the correct translation initiation codon (nucleotides 267–269 in SEQ. ID. No:9), and because the use of the upstream ATG sequence to initiate translation of the  $\beta_2$  DNA would result in the generation of an inoperative peptide (because the upstream ATG is not in the correct reading frame), an additional  $\beta_2$ -encoding construct was prepared as follows. A 2.2 kb KspI/EcoRI DNA fragment containing nucleotides 262–2450 of SEQ. ID. No:9 was ligated to pCMV-T7-2 in operative association with the T7 promoter of the plasmid to create pCMV-KE $\beta_2$ . The  $\beta_2$  subunit DNA contained in pCMV-KE $\beta_2$  retains only 5 nucleotides of 5' untranslated sequence upstream of the correct translation initiation codon.

## EXAMPLE 3

## Expression of Recombinant Human Nicotinic AChR in Oocytes

*Xenopus* oocytes were injected with in vitro transcripts prepared from constructs containing DNA encoding  $\alpha_3$ ,  $\alpha_7$ ,  $\beta_2$  and  $\beta_4$  subunits. Electrophysiological measurements of the oocyte transmembrane currents were made using the two-electrode voltage clamp technique (see, e.g., Stuhmer (1992) *Meth. Enzymol.* 207:319–339).

## 1. Preparation of in vitro Transcripts

Recombinant capped transcripts of pCMV-KE $\alpha_3$ , pCMV-KE $\beta_2$ , KE $\beta_4$ .6/pGEM and pCMV-KE $\beta_4$  were synthesized from linearized plasmids using the mCAP RNA Capping Kit (Cat. #200350 from Stratagene, Inc., La Jolla, Calif.). Recombinant capped transcripts of pCMV-KE $\alpha_7$ , pCMV-KE $\alpha_7$ .3 and H $\beta_2$ .1F were synthesized from linearized plasmids using the MEGAscript T7 in vitro transcription kit according to the capped transcript protocol provided by the manufacturer (Catalog #1334 from AMBION, Inc., Austin, Tex.). The mass of each synthesized transcript was determined by UV absorbance and the integrity of each transcript was determined by electrophoresis through an agarose gel.

## 2. Electrophysiology

*Xenopus* oocytes were injected with either 12.5, 50 or 125 ng of human nicotinic AChR subunit transcript per oocyte. The preparation and injection of oocytes were carried out as described by Dascal (1987) in *Crit. Rev. Biochem.* 22:317–387. Two-to-six days following mRNA injection, the oocytes were examined using the two-electrode voltage clamp technique. The cells were bathed in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.3) containing 1  $\mu$ M atropine with or without 100  $\mu$ M d-tubocurarine. Cells were voltage-clamped at –60 to –80 mV. Data were acquired with Axotape software at 2–5 Hz. The agonists acetylcholine (ACh), nicotine, and cytisine were added at concentrations ranging from 0.1  $\mu$ M to 100  $\mu$ M. The results of electrophysiological analyses of the oocytes are summarized in Table 1.

TABLE 1

Template, ng RNA Injected	Number of oocytes responding	Current Agonists	Amplitude
pCMV-KE $\alpha$ 3, 12.5 ng	0 of 8	ACh, Nicotine	
KE $\beta$ 4.6/pGEM, 12.5 ng	0 of 9	ACh, Nicotine	
pCMV-KE $\alpha$ 3, 12.5 ng + KE $\beta$ 4.6/pGEM, 12.5 ng	4 of 5	ACh, Nicotine	20–550 nA
pCMV-KE $\alpha$ 3, 12.5 ng + KE $\beta$ 4.6/pGEM, 12.5 ng	3 of 4	ACh, Cytisine, Nicotine	20–300 nA
pCMV-KE $\alpha$ 3, 125 ng + pCMV-KE $\beta$ 4, 125 ng	5 of 5	Ch, Nicotine, Cytisine	200–500 nA
pCMV-KE $\alpha$ 3, 125 ng + pCMV-KE $\beta$ 4, 125 ng	6 of 6	ACh, Nicotine, Cytisine	100–400 nA
pCMV-KE $\alpha$ 7.3, 125 ng	3 of 15	ACh	~20 nA
pCMV-KE $\alpha$ 7, 125 ng	11 of 11	ACh	20–250 nA
pCMV-KE $\alpha$ 3, 12.5 ng + pCMV-KE $\beta$ 2, 12.5 ng	2 of 9	ACh, Nicotine	<10 nA
pCMV-KE $\alpha$ 3, 125 ng + pCMV-KE $\beta$ 2, 125 ng	0 of 9	ACh, Nicotine	
pCMV-KE $\alpha$ 3, 125 ng + H $\beta$ 2.1 F, 125 ng	13 of 16	ACh (100 $\mu$ M), ACh (300 $\mu$ M)	~20 nA, ~80 nA

a. Oocytes Injected with  $\alpha_3$  and/or  $\beta_4$  Transcripts

Oocytes that had been injected with 12.5 ng of the  $\alpha_3$  transcript or 12.5 ng of the  $\beta_4$  transcript did not respond to application of up to 100  $\mu$ M ACh, nicotine or cytisine. Thus, it appears that these subunits do not form functional homomeric nicotinic AChR channels. By contrast, oocytes injected with 12.5 or 125 ng of the  $\alpha_3$  transcript and 12.5 ng or 125 ng of the  $\beta_4$  transcript exhibited detectable inward currents in response to ACh, nicotine, and cytisine at the tested concentrations (0.1  $\mu$ M to 10  $\mu$ M). Some differences in the kinetics of the responses to cytisine compared to nicotine and ACh were observed. The relative potency of the agonists appeared to be cytisine>ACh>nicotine, which differs from the results of similar studies of oocytes injected with transcripts of the rat nicotinic AChR  $\alpha_3$  and  $\beta_4$  subunits (see, for example, Luetje et al. (1991) *J. Neurosci.* 11:837–845).

The responses to ACh and nicotine were reproducibly blocked by d-tubocurarine. For example, complete blockage of the response to ACh was observed in the presence of 100  $\mu$ M d-tubocurarine. The inhibition appeared to be reversible. The responses to ACh, nicotine and cytisine were also at least partially blocked by 100 nM mecamylamine.

The current response of  $\alpha_3$ - $\beta_4$ -injected oocytes to 10  $\mu$ M ACh was also examined in terms of membrane voltage. In these experiments, voltage steps were applied to the cells in the presence of ACh. The graph of current vs. voltage appeared typical of responses observed for Na<sup>+</sup>, K<sup>+</sup>-permeable channels. For example, the zero current level (reversal potential) is less than –40 mV. The contribution of Ca<sup>++</sup> flux to the total current can be ascertained by varying the calcium concentration in the external medium and taking multiple current measurements at different holding potentials around the reversal potential. Such studies indicate that the channel carrying the current generated in response to ACh treatment of  $\alpha_3$ - $\beta_4$ -injected oocytes is permeable to Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>++</sup>.

b. Oocytes Injected with  $\alpha_7$  Subunit Transcripts

As described in Example 2, two constructs were prepared for use in expressing the human neuronal nicotinic AChR  $\alpha_7$  subunit. Plasmid pCMV-KE $\alpha$ 7.3 contains the  $\alpha_7$  subunit coding sequence with 72 nucleotides of 5' untranslated sequence upstream of the translation initiation codon. Plasmid pCMV-KE $\alpha$ 7 contains the  $\alpha_7$  subunit coding sequence devoid of any 5' untranslated sequence and further contains a consensus ribosome binding site immediately upstream of the coding sequence.

Oocytes injected with 125 ng of  $\alpha_7$  transcript synthesized from pCMV-KE $\alpha$ 7 displayed inward currents in response to 10 or 100  $\mu$ M ACh. This response was blocked by 100  $\mu$ M d-tubocurarine.

Oocytes injected with 125 ng of  $\alpha_7$  transcript synthesized from pCMV-KE $\alpha$ 7.3 exhibited ACh-induced currents that were substantially weaker than those of oocytes injected with  $\alpha_7$  transcript synthesized from pCMV-KE $\alpha$ 7. These results indicate that human neuronal nicotinic AChR  $\alpha_7$  subunit transcripts generated from  $\alpha_7$  subunit DNA containing a ribosome binding site in place of 5' untranslated sequence may be preferable for expression of the  $\alpha_7$  receptor in oocytes.

c. Oocytes Injected with  $\beta_3$  and  $\beta_2$  Subunit Transcripts

As described in Example 2, two constructs were prepared for use in expressing the human neuronal nicotinic AChR  $\beta_2$  subunit. Plasmid H $\beta$ 2.1F contains the  $\beta_2$  subunit coding sequence with 266 nucleotides of 5' untranslated sequence upstream of the translation initiation codon. Plasmid pCMV-KE $\beta$ 2 contains the  $\beta_2$  subunit coding sequence and only 5 nucleotides of 5' untranslated sequence upstream of the translation initiation codon.

Oocytes injected with transcripts of pCMV-KE $\alpha$ 3 and pCMV-KE $\beta$ 2 displayed no current in response to nicotinic AChR  $\alpha_3$  agonists. In contrast, oocytes injected with transcripts of pCMV-KE $\alpha$ 3 and H $\beta$ 2.1F displayed ~20 nA inward currents in response to 100  $\mu$ M ACh and ~80 nA inward currents in response to 300  $\mu$ M ACh. The current response was blocked by 100  $\mu$ M d-tubocurarine. These results indicate that human neuronal nicotinic AChR  $\beta_2$  subunit transcripts generated from  $\beta_2$  subunit DNA containing 5' untranslated sequence may be preferable to transcripts generated from  $\beta_2$  DNA containing only a small portion of 5' untranslated sequence for expression of the  $\alpha_3\beta_2$  receptors in oocytes.

## EXAMPLE 4

## Recombinant Expression of Human nNACHR Subunits in Mammalian Cells

1. Recombinant Expression of Human NACHR  $\alpha_3$  and  $\beta_4$  or  $\alpha_7$  Subunits in HEK 293 Cells:

Human embryonic kidney (HEK) 293 cells were transiently and stably transfected with DNA encoding human

neuronal nicotinic AChR  $\alpha_3$  and  $\beta_4$ , or  $\alpha_7$  subunits. Transient transfectants were analyzed for expression of nicotinic AChR using various assays, e.g., electrophysiological methods,  $\text{Ca}^{2+}$ -sensitive fluorescent indicator-based assays and [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin-binding assays.

#### 1. Transient Transfection of HEK Cells

Two transient transfection were performed. In one transfection, HEK cells were transiently co-transfected with DNA encoding  $\alpha_3$  (plasmid pCMV-KE $\alpha_3$ ) and  $\beta_4$  (plasmid pCMV-KF $\beta_4$ ) subunits. In the other transfection, HEK cells were transiently transfected with DNA encoding the  $\alpha_7$  subunit (plasmid pCMV-KE $\alpha_7$ ). In both transfections,  $\sim 2 \times 10^6$  HEK cells were transiently transfected with 18  $\mu\text{g}$  of the indicated plasmid(s) according to standard  $\text{CaPO}_4$  transfection procedures [Wigler et al. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:1373–1376]. In addition, 2  $\mu\text{g}$  of plasmid pCMV $\beta$ gal (Clontech Laboratories, Palo Alto, Calif.), which contains the *Escherichia coli*  $\beta$ -galactosidase gene fused to the CMV promoter, were co-transfected as a reporter gene for monitoring the efficiency of transfection. The transfectants were analyzed for  $\beta$ -galactosidase expression by measurement of  $\beta$ -galactosidase activity [Miller (1972) *Experiments in Molecular Genetics*, pp. 352–355, Cold Spring Harbor Press]. Transfectants can also be analyzed for  $\beta$ -galactosidase expression by direct staining of the product of a reaction involving  $\beta$ -galactosidase and the X-gal substrate [Jones (1986) *EMBO* 5:3133–3142].

The efficiency of transfection of HEK cells with pCMV-KE $\alpha_3$ /pCMV-KE $\beta_4$  was typical of standard efficiencies, whereas the efficiency of transfection of HEK cells with pCMV-KE $\alpha_7$  was below standard levels.

#### 2. Stable Transfection of HEK Cells

HEK cells were transfected using the calcium phosphate transfection procedure [*Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1–9.1.9 (1990)]. Ten-cm plates, each containing one-to-two million HEK cells were transfected with 1 ml of DNA/calcium phosphate precipitate containing 9.5  $\mu\text{g}$  pCMV-KE $\alpha_3$ , 9.5  $\mu\text{g}$  pCMV-KE $\beta_4$  and 1  $\mu\text{g}$  pSV2neo (as a selectable marker). After 14 days of growth in media containing 1  $\mu\text{g}/\text{ml}$  G418, colonies had formed and were individually isolated by using cloning cylinders. The isolates were subjected to limiting dilution and screened to identify those that expressed the highest level of nicotinic AChR, as described below.

#### 3. Analysis of Transfectants

##### a. Fluorescent Indicator-based Assays

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including  $\text{Ca}^{++}$ , through the receptor channel.  $\text{Ca}^{++}$  entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic  $\text{Ca}^{++}$  levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol.

Interaction of the free indicator with calcium results in increased fluorescence of the indicator, therefore, an increase in the intracellular  $\text{Ca}^{2+}$  concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying icotinic AChR has been described in commonly assigned pending U.S. patent application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

HEK cells that were transiently or stably co-transfected with DNA encoding  $\alpha_3$  and  $\beta_4$  subunits were analyzed for expression of functional recombinant nicotinic AChR using the automated fluorescent indicator-based assay. The assay procedure was as follows.

Untransfected HEK cells (or HEK cells transfected with pCMV-T7-2) and HEK cells that had been co-transfected with pCMV-KE $\alpha_3$  and pCMV-KE $\beta_4$  were plated in the wells of a 96-well microtiter dish and loaded with fluo-3 by incubation for 2 hours at 20° C. in a medium containing 20  $\mu\text{M}$  fluo-3, 0.2% Pluronic F-127 in HBS (125 mM NaCl, 5 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.62 mM  $\text{MgSO}_4$ , 6 mM glucose, 20 mM HEPES, pH 7.4). The cells were then washed with assay buffer (i.e., HBS). The antagonist d-tubocurarine was added to some of the wells at a final concentration of 10  $\mu\text{M}$ . The microtiter dish was then placed into a fluorescence plate reader and the basal fluorescence of each well was measured and recorded before addition of 200  $\mu\text{M}$  nicotine to the wells. The fluorescence of the wells was monitored repeatedly during a period of approximately 60 seconds following addition of nicotine.

The fluorescence of the untransfected HEK cells (or HEK cells transfected with pCMV-T7-2) did not change after addition of nicotine. In contrast, the fluorescence of the co-transfected cells, in the absence of d-tubocurarine, increased dramatically after addition of nicotine to the wells. This nicotine-stimulated increase in fluorescence was not observed in co-transfected cells that had been exposed to the antagonist d-tubocurarine. These results demonstrate that the co-transfected cells express functional recombinant AChR that are activated by nicotine and blocked by d-tubocurarine.

##### b. $\alpha$ -Bungarotoxin Binding Assays

HEK293 cells transiently transfected with pCMV-KE $\alpha_7$  were analyzed for [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin (BgTx) binding. Both whole transfected cells and membranes prepared from transfected cells were examined in these assays. Rat brain membranes were included in the assays as a positive control.

Rat brain membranes were prepared according to the method of Hampson et al. (1987) *J. Neurochem* 49:1209. Membranes were prepared from the HEK cells transfected with pCMV-KE $\alpha_7$  and HEK cells transiently transfected with plasmid pUC19 only (negative control) according to the method of Perez-Reyes et al. (1989) *Nature* 340:233. Whole transfected and negative control cells were obtained by spraying the tissue culture plates with phosphate-buffered saline containing 0.1% (w/v) BSA. The cells were then centrifuged at low speed, washed once, resuspended in assay buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 20 mM HEPES, 0.1% (w/v)BSA, 0.05% (w/v) bacitracin and 0.5 mM PMSF, pH 7.5) and counted.

Specific binding of [ $^{125}\text{I}$ ]- $\alpha$ -BgTx to rat brain membranes was determined essentially as described by Marks et al. (1982) *Molec. Pharmacol.* 22:554–564, with several modifications. The membranes were washed twice in assay buffer. The assay was carried out in 12x75 mm polypropylene test tubes in a total volume of 0.5 ml assay buffer. The membranes were incubated with 10 nM [ $^{125}\text{I}$ ]- $\alpha$ -BgTx (New England Nuclear, Boston, Mass.) for one hour at 37° C. The

assay mixtures were then centrifuged at 2300×g for 10 minutes at 4°C. The supernatant was decanted and the pellets were washed twice with 2 ml aliquots of ice-cold assay buffer. The supernatants were decanted again and the radioactivity of the pellets was measured in a  $\gamma$ -counter. Non-specific binding was determined in the presence of 1  $\mu$ M unlabeled  $\alpha$ -BgTx. Specific binding was determined by subtracting nonspecific binding from total binding. Specific binding of [<sup>125</sup>I]- $\alpha$ -BgTx to membranes prepared from transfected and negative control cells was determined as described for determining specific binding to rat brain membranes except that the assay buffer did not contain BSA, bacitracin and PMSF. Specific binding of [<sup>125</sup>I]- $\alpha$ -BgTx to transfected and negative control whole cells was determined basically as described for determining specific binding to rat brain membranes.

[<sup>125</sup>I]- $\alpha$ -BgTx binding was evaluated as a function of membrane concentration and as a function of incubation time. [<sup>125</sup>I]- $\alpha$ -BgTx binding to rat brain membranes increased in a linear fashion with increasing amounts of membrane (ranging between 25–500  $\mu$ g). The overall signal-to-noise ratio of binding (i.e., ratio of total binding to non-specific binding) was 3:1. Although some binding of [<sup>125</sup>I]- $\alpha$ -BgTx to transfected cell membranes was detected, it was mostly non-specific binding and did not increase with increasing amounts of membrane. [<sup>125</sup>I]- $\alpha$ -BgTx binding to the transfectants and negative control cells appeared to be similar.

To monitor [<sup>125</sup>I]- $\alpha$ -BgTx binding to rat brain membranes and whole transfected and negative control cells, 300  $\mu$ g of membrane or 500,000 cells were incubated with 1 nM or 10 nM [<sup>125</sup>I]- $\alpha$ -BgTx, respectively, at 37° C. for various times ranging from 0–350 min. Aliquots of assay mixture were transferred to 1.5 ml microfuge tubes at various times and centrifuged. The pellets were washed twice with assay buffer. [<sup>125</sup>I]- $\alpha$ -BgTx binding to rat brain membranes increased with time and was maximal after three hours. The binding profiles of the transfected and negative control cells were the same and differed from that of rat brain membranes.

#### Recombinant Expression of Human nAChR Subunits (Multimeric Subunit Combinations) in Mammalian Cells

##### II. (a) Preparation of Constructs for the Expression of Recombinant Human Neuronal Nicotinic nAChR Containing Multimeric Subunits.

Isolated cDNAs encoding human neuronal nAChRs were incorporated into vectors for use in expressing the subunits in mammalian host cells.

##### A. Construct for Expression of a Human nAChR $\alpha$ 3 Subunit.

Construct pCMV-KE $\alpha$ 3 (FIG. 12) is described in U.S. Pat. No. 5,837,489, the contents of which are incorporated by reference herein in their entirety, was digested with HindIII and NotI to release a 1.7 kb DNA fragment containing the entire  $\alpha$ 3 coding region. The expression construct pcDNA3-KE $\alpha$ 3 was prepared by ligating the 1.7 kb  $\alpha$ 3 DNA fragment from pCMV-KE $\alpha$ 3 into vector HindIII and NotI digested pcDNA3 (Invitrogen).

##### B. Construct for Expression of a Human nAChR $\alpha$ 5 Subunit.

DNA fragments encoding portions of a human nAChR  $\alpha$ 5 subunit were ligated together to generate a full-length  $\alpha$ 5 subunit coding sequence contained in plasmid pcDNA1/Amp-KE $\alpha$ 5.5F. This construct was modified by replacing the 5' untranslated sequence of the  $\alpha$ 5 subunit DNA with a consensus ribosome binding site, RBS, (5'-GCCACC-3', see Kozak (1987) Nucl. Acids Res. 15:8225–8148) to generate pcDNA1/Amp-KE $\alpha$ 5RBS. Construct pcDNA1/Amp-

KE $\alpha$ 5RBS was digested with BamHI and EcoRI to release a 1.7 kb DNA fragment containing the consensus ribosome binding site immediately 5' to the translation initiation codon of  $\alpha$ 5 and also containing the entire  $\alpha$ 5 coding region.

Construct pcDNA3-KE $\alpha$ 5RBS was prepared by digestion of pcDNA3 with BamHI and EcoRI followed by ligation of the 1.7 kb  $\alpha$ 5 DNA fragment. The pcDNA3-KE $\alpha$ 5RBS construct was then digested with Asp718I and BstXI to release a 1.7 kb fragment containing the entire  $\alpha$ 5 coding sequence with the RBS immediately 5' to the  $\alpha$ 5 sequence. This fragment was ligated into expression vector pHOOK3 (Invitrogen) which had been digested with Asp718I and BstXI to generate the expression construct pHOOK3-KE $\alpha$ 5RBS (FIG. 13).

##### C. Construct for Expression of a Human nAChR $\beta$ 2 Subunit.

Construct pCMV-KE $\beta$ 2 (described in U.S. Pat. No. 5,910,582) was modified by replacing the 5' untranslated sequence of the  $\beta$ 2 subunit DNA with a consensus ribosome binding site (5'-GCCACC-3', see Kozak (1987) Nucl. Acids Res. 15:8225–8148) to generate pCMV-KE $\beta$ 2RBS. The expression vector pCMV-KE $\beta$ 2RBS was digested with BglII and EcoRI to release a 2.2 kb DNA fragment containing the consensus ribosome binding site immediately 5' to the translation initiation codon of  $\beta$ 2 and also containing the entire  $\beta$ 2 coding region. This 2.2 kb DNA fragment was ligated into expression vector pcDNA3 that had been digested with BamHI and EcoRI. The BamHI site is compatible with BglII and this ligation generated expression construct pcDNA3-KE $\beta$ 2RBS (FIG. 14).

##### II (b) Recombinant Expression of the Human $\alpha$ 3 $\beta$ 2 $\alpha$ 5 nAChR in HEK293 Cells.

Human embryonic kidney cells (HEK 293) were stably co-transfected with DNA encoding human neuronal nAChR  $\alpha$ 3,  $\beta$ 2 and  $\alpha$ 5 and analyzed for expression of nAChRs using various assays, for example, calcium sensitive fluorescent indicator-based assays and electrophysiological methods.

##### 1. Stable Co-transfection of HEK293 Cells with Human $\alpha$ 3, $\beta$ 2 and $\alpha$ 5 nAChRs.

###### a. Expression Strategy.

The  $\alpha$ 5 nAChR is non-functional when expressed with either another  $\alpha$  subunit or another  $\beta$  subunit. In order to develop a functional 3-way nAChR that includes the  $\alpha$ 5 subunit,  $\alpha$ 5 was co-expressed with both  $\alpha$ 3 and  $\beta$ 2. The antibiotic selection strategy was designed to take advantage of the lack of function of co-expression of either  $\alpha$ 3 $\alpha$ 5 or  $\alpha$ 5 $\beta$ 2. Even though these combinations would survive the antibiotic selection, they would be non-functional. Using this expression strategy, the only possible nAChR subunit combination surviving antibiotic selection and having functional responses would be  $\alpha$ 3 $\beta$ 2 $\alpha$ 5. The expression strategy for the generation of this subunit combination is described in detail below.

The  $\alpha$ 3 was cloned into pcDNA3 (Invitrogen) that encodes a neomycin resistance gene permitting tolerance to the antibiotic G418. The  $\beta$ 2 subunit was also cloned into pcDNA3. The  $\alpha$ 5 subunit was cloned into the expression vector pHOOK3 (Invitrogen) which encodes the Zeocin™ (Invitrogen) resistance gene that allows tolerance to the antibiotic Zeocin™. By this strategy, cells stably expressing the  $\alpha$ 5 nAChR and  $\alpha$ 3 or  $\alpha$ 5 and  $\beta$ 2 could survive in a selection culture medium containing both G418 and Zeocin™. However, stable expression of  $\alpha$ 3,  $\alpha$ 5 and  $\beta$ 2 would be required for function.

###### b. Recombinant Expression of Human $\alpha$ 3 $\beta$ 2 $\alpha$ 5 nAChRs.

HEK293 cells were stably co-transfected with DNA encoding human neuronal nAChRs  $\alpha$ 3,  $\beta$ 2 and  $\alpha$ 5 using the lipofection transfection procedure (Current Protocols in



Molecular Biology, Volume 1, 9.4.1–9.4.5 and 9.5.1–9.5.6, the contents of which are incorporated by reference herein). HEK293 cells were harvested and plated onto 10 cm tissue culture plates that were coated with poly-D-lysine. The HEK293 cells were plated at a concentration of 1.2 million 5 cells per plate, 24 hours prior to transfection. Two micrograms of DNA encoding  $\alpha 3$  (mammalian expression vector pcDNA3-KE $\alpha 3$ ), 2  $\mu\text{g}$  of DNA encoding  $\beta 2$  (pcDNA3-KE $\beta 2$ RBS) and 2  $\mu\text{g}$  of DNA encoding  $\alpha 5$  (pHOOK3-KE $\alpha 5$ RBS) were diluted in 300  $\mu\text{l}$  of Dulbecco's Modified Eagle Medium (DMEM) and combined with 20  $\mu\text{l}$  of LipofectAMINE™ Reagent (Gibco-BRL) for 15 minutes. The HEK293 cells were washed twice with DMEM. This DNA/LipofectAMINE mixture was further diluted into 5.3 ml of DMEM and overlaid onto the HEK293 cells. The overlaid cells were incubated for 5 hours in an incubator at 37° C., in a humidified atmosphere containing 5% carbon dioxide. Cell plates were washed twice with 5 mls of complete media (DMEM, 6% iron-supplemented calf serum, 2 mM glutamine, 100 units per ml of penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin) then overlaid with 10 ml of complete medium and placed in an incubator for 48 hours.

Forty-eight hours post-transfection, cell plates were split at a 1:4 ratio, generating four culture plates. Twenty hours later, complete medium containing 100  $\mu\text{g}/\text{ml}$  of G418 plus 40  $\mu\text{g}/\text{ml}$  Zeocin™ was added to the cells for 14 days. Medium was replaced every 2 to 4 days. After this period, colonies had formed on the plates and were isolated using trypsin-soaked circles of sterile filter paper. 24 isolates were cultured, 20 survived and were expanded for functional assay using fluorescence-based measurements of internal calcium concentrations (Reference to analysis of transfectants, section 2). Two parental cell lines, 83-13 and 83-19 exhibited robust expression of the 3-way combination in functional calcium assays and both were subcloned by limiting dilution.

Thirty seven subclones from parental cell line 83-19 were screened in the fluorescence-based calcium assay. Sixteen subclones were positive in this assay and showed epibatidine-induced increases in internal calcium. Twelve subclones from parental cell line 83-13 were screened in the fluorescence-based calcium assay and five subclones were positive. Four subclones, including subclone 83-19-15 were selected based on activity in calcium assays.

83-19-15 was further subcloned by limiting dilution, and 18 subclones were screened for acetylcholine-induced increases in internal calcium. Four subclones (83-19-15-26, 83-19-15-27, 83-19-15-42 and 83-19-15-48) were selected based on a positive functional response in this assay. These subclones then entered a stability study where they were monitored for acetylcholine-induced increases in internal calcium at two-weekly intervals for approximately 15 weeks.

Subclone 83-19-15-27 was selected based on the stable functional response to low doses of acetylcholine (1  $\mu\text{M}$ ) observed during the stability study. This cell line was confirmed to have acceptable responses as a random screening target in the high throughput screening assay and renamed A3B2A5 after validation in this assay (example 5, protocol A).

## 2. Analysis of Transfectants

### a. Fluorescent Indicator-based Assays

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including  $\text{Ca}^{++}$ , through the receptor channel.  $\text{Ca}^{++}$  entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the

channel can also result in an increase in cytoplasmic  $\text{Ca}^{++}$  levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular  $\text{Ca}^{2+}$  concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying nicotinic AChR has been described in commonly assigned pending U.S. patent application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

HEK293 cells that were stably transfected with DNA encoding the human  $\alpha 3\beta 2\alpha 5$  subunit were analyzed for expression of functional recombinant nAChRs using the automated fluorescent indicator-based assay.

Briefly, untransfected HEK293 cells and HEK293 cells that had been transfected with DNA encoding human  $\alpha 3$ ,  $\alpha 5$  and  $\beta 2$  nAChRs were plated in the wells of a poly-D-lysine coated 96-well microtiter dish at a cell density of 75,000 to 200,000 cells per well. Cells were grown in an incubator at 37° C. for 2–3 hours, then transferred to an incubator maintained at 28° C. Forty-eight hours after plating, cell culture medium was decanted and cells washed with an assay buffer (HBK) containing 155 mM NaCl, 4.6 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 21.8 mM  $\text{CaCl}_2$ , 1  $\mu\text{M}$  atropine, 6 mM glucose and 20 mM HEPES-NaOH pH7.4. Washed cells were incubated with 20  $\mu\text{M}$  fluo-3-acetoxymethylester containing 0.16% pluronic F-127 at 22° C. for 2 hours in the dark. Dye not taken up by cells was removed by aspiration followed by washing with 250  $\mu\text{l}$  HBK. Fluorescence measurements were performed at 0.33 sec intervals using a 96-well microtiter plate-reading fluorometer (Cambridge Technology, Inc.).

Ten basal fluorescence readings were recorded prior to addition of agonist (either 100 nM epibatidine, or 1  $\mu\text{M}$  acetylcholine). Responses after the addition of epibatidine were recorded for approximately 60 sec. Maximal fluorescence ( $F_{max}$ ) was determined after lysing the cells with 0.25% Triton X-100, and minimal fluorescence ( $F_{min}$ ) was determined after subsequent quenching with 10 mM  $\text{MnCl}_2$ . Calculation of  $[\text{Ca}^{2+}]_i$  was performed as described by Kao et al. (1989). Cellular responses were quantitated by calculating either the ratio of peak  $[\text{Ca}^{2+}]_i$  after agonist addition to the basal  $[\text{Ca}^{2+}]_i$  prior to agonist addition, or by the difference between peak  $[\text{Ca}^{2+}]_i$  and basal  $[\text{Ca}^{2+}]_i$ .

The fluorescence of the untransfected HEK cells did not change after addition of nicotine. In contrast, the fluorescence of the co-transfected cells, in the absence of d-tubocurarine, increased dramatically after addition of nicotine to the wells. This nicotine-stimulated increase in fluorescence was not observed in co-transfected cells that had been exposed to the antagonist d-tubocurarine. These results demonstrate that the co-transfected cells express the above referenced functional recombinant multimeric AChR subunit combination that were activated by nicotine and blocked by d-tubocurarine.

b. Characteristics of the Stable Cell Line A3B2A5 that Expresses the Human  $\alpha 3\beta 2\alpha 5$  nAChR.

Pharmacological analysis of agonist-induced increases in internal calcium using the fura-2 calcium assay (Protocol A, *infra*, Reference to SpeedReader patent?) showed the expression of two populations of nAChRs in A3B2A5 cells: one population displayed high sensitivity to some nAChR agonists while the second showed a sensitivity to agonists indistinguishable from that observed in cell line A3B2 (which expresses human  $\alpha 3\beta 2$  nAChRs). The high affinity site in A3B2A5 cells displays a 200- to 6000-fold lower  $EC_{50}$  value for the agonists acetylcholine (ACh), nicotine and cytosine compared to  $\alpha 3\beta 2$  nAChRs. FIGS. 9a and 9b illustrate some of the pharmacology of the A3B2A5 cell line. The changes in agonist sensitivity result in a rank order of agonist potency for A3B2A5 that differs from that of A3B2 and thus demonstrates the presence of a novel receptor ( $\alpha 3\beta 2\alpha 5$ ) in cell line A3B2A5. In whole-cell voltage-clamped A3B2A5 cells, we found that the desensitization kinetics of currents elicited by low doses of ACh are significantly slower in A3B2A5 cells than A3B2 cells (Protocol B) (FIG. 10). The differences in biophysical properties of A3B2A5 and A3B2 also indicate the expression of a novel receptor, the  $\alpha 3\beta 2\alpha 5$  nAChR, in cell line A3B2A5 and these are illustrated in FIG. 9b. The homogeneity of the cell line was verified by single-cell imaging of agonist-induced increases in intracellular free calcium concentration (Protocol C). Co-precipitation experiments demonstrated the co-assembly of the  $\alpha 5$  nAChR with  $\alpha 3$  and with  $\beta 2$  (protocol D, FIG. 11).

The protocols for the above referenced data is presented hereafter.

#### A. Fluorescence-based Calcium Assays Using Fura-2.

A cell line, A3B2A5, stably transfected with DNAs encoding human  $\alpha 3$ ,  $\alpha 5$ , and  $\beta 2$  receptors is plated in black-walled 96-well plates, grown 2 to 3 hours at 37° C. and then 2 days at 28° C. At the start of the assay, assay the plates are washed with in HEPES buffered saline (HBS) containing 1  $\mu$ M atropine (HBSA) (wash cycle=aspirate, dispense $\times$ 3) to leave 180  $\mu$ l residual HBSA per well. Then a background measurement of a sample plate is taken by the SpeedReader for 20 frames alternating the excitation light between 350 and 385 nm at four hertz. Twenty  $\mu$ l of 10  $\mu$ M fura-2 dye containing is then added to each well and incubated with the cells at ambient temperature for one hour to two hours. After dye loading the free dye is washed from the wells with HBSA to leave 180  $\mu$ l residual buffer per well. Two minutes after washing, a kinetic reading is taken while the test chemicals are added. The test compounds are prepared in HBSA containing 80 mM  $CaCl_2$  and 1% DMSO. The kinetic reading is composed of 140 frames, alternating between 350 and 385 as in the 20 frame background reading. However, the first 20 frames of the kinetic reading are taken before test chemical addition. The difference between these 20 frames and the background give the fluorescence due to the Ca-indicating dye fura-2. After the first 20 frames are collected 20  $\mu$ l of the test compound is dispensed from a 96-channel pipettor to the entire plate at once without halting the reading. The remainder of the 120 frames of data measure the response.

Absolute Ca concentrations are not calculated from these readings, rather the directly measured fluorescence ratio is used as a surrogate for Ca. The fluorescence ratio is calculated as dye fluorescence generated by excitation at 350 nm divided by dye fluorescence generated by excitation at 385 nm. The raw activity in a well is calculated as the maximum fluorescence ratio after compound addition divided by the average fluorescence ratio before compound addition.

#### B. Electrophysiological Analysis

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques.

HEK293 cells stably transfected with DNA encoding the human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  subunits were analyzed electrophysiologically for the presence of nAChR agonist-dependent currents. HEK293 cells stably expressing human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  nAChRs were plated at a density of  $1.5 \times 10^5$  cells/35-mm dish on poly-D-lysine-coated glass coverslips (0.1 mg/ml, SIGMA) and incubated at 37° C. for 2–3 hours, then for 48 hours at 28° C. Recordings were performed with an Axopatch 200A amplifier (Axon Instruments) using the whole-cell voltage-clamp configuration. Membrane potential was held at  $-100$  mV. The standard external recording solution (mammalian Ringer's) consisted of (in mM) 160 NaCl, 5 KCl, 2  $CaCl_2$ , 1  $MgCl_2$ , 11 glucose, 0.001 atropine, and 5 HEPES, pH 7.3. Ringer's solution was superfused at a rate of  $\approx 3.0$  ml/min (110  $\mu$ l recording chamber). The recording pipette solution was composed of 135 mM CsCl, 10 mM EGTA, 1 mM  $MgCl_2$  and 10 mM HEPES, pH 7.3 (with or without 4 mM Mg-ATP). Experiments were performed at room temperature. Agonist, dissolved in Ringer's solution, was applied for 200–500 ms using a fast application system, consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezoelectric drive, Winston Electronics). The speed of solution exchange between control and nicotine-containing solutions, measured as the open-tip response, displays a time constant  $\tau = 0.7$  ms, with steady state reached  $< 3$  ms. Data were digitized at 6.7 kHz and filtered at 2 kHz on line. Data analysis was performed using pClamp software (Axon Instruments).

#### B. Single Cell Calcium Imaging Assays Using Fura-2

Cells stably transfected with DNAs encoding human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  nAChR subunits were plated on poly-D-lysine-coated glass coverslips at a density of  $3 \times 10^5$  cells/35 mm dish and grown at 28° C. Forty-eight hours later, imaging experiments were performed at room temperature, using a Nikon TE200 inverted microscope attached to a DeltaRAM imaging System (Photon Technology International). Cells were incubated with 1  $\mu$ M fura-2-AM (Molecular Probes, Inc.) for 0.5–1 h and washed with mammalian Ringer's solution (see example 4, 2c for composition) to remove excess dye. Cells were transferred to a recording chamber (110  $\mu$ l, Warner Instruments), and continuously superfused with HBK containing 21.8 mM  $CaCl_2$  and 1  $\mu$ M atropine at a rate of 8–10 ml/min. Agonist was applied by switching between reservoirs. Cells were alternatively excited at 360 and 381 nm (0.5 Hz) to determine ratio images.

#### C. Western Analysis and Immunoprecipitation to Demonstrate Co-expression of $\alpha 3$ , $\beta 2$ and $\alpha 5$ nAChR Subunit Proteins.

Cells stably transfected with DNA encoding human  $\alpha 3$ ,  $\beta 2$  and  $\alpha 5$  nAChR subunits were harvested from 10-cm plates and washed with phosphate-buffered saline (PBS; 140

mM NaCl, 3 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). Washed cells were resuspended in 50 mM Tris pH 7.4, 1 mM EDTA containing a cocktail of protease inhibitors (Complete™, Boehringer Mannheim, Indianapolis, Ind.) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000×g for 5 min to remove cellular debris, and the supernatant fraction was centrifuged at 100,000×g for 120 min to pellet the membranes. The membranes were resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1% SDS) containing protease inhibitor cocktail.

For immunoprecipitation experiments, 200 μg of membranes were immunoprecipitated with 20 μg of a sheep anti-rat α3 polyclonal antibody (Bethyl Laboratories, or 2 μg a rabbit anti-human β2 polyclonal antibody (MRL San Diego) overnight at 4° C. The antibody-antigen complexes were affinity-purified using Protein G sepharose, incubated overnight at 4° C. then solubilized in SDS sample buffer. For immunoblot analysis, membranes were solubilized in Tris-Glycine SDS Sample Buffer (Novex) containing 5% 2-mercaptoethanol and heated at 65° C. for 10 min. Solubilized proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hy-Bond ECL, Amersham, Arlington Heights, Ill.). Blots were rinsed once in PBS, 0.1% Tween-20 (wash buffer), then blocked for 3 h in 5% Carnation non-fat dry milk dissolved in wash buffer (blocking buffer).

The human α5 protein was detected with a sheep anti-rat α5 antibody (Bethyl Laboratories). The α5 antibody was diluted to 15 μg/ml in blocking buffer and incubated with the nitrocellulose membrane for 3 h at room temperature. The membranes were washed three times in wash buffer. The secondary antibody was peroxidase-conjugated donkey anti-sheep IgG (Cappel Antibodies) diluted 1:1000 in blocking buffer and incubated with membranes for 45 min at room temperature, followed by five changes of wash buffer. The antibody signal was visualized using the ECL developing system (Amersham) according to the manufacturer's directions.

The above strategy may be employed in expressing any one of the following multimeric subunit combinations of the alpha and beta subunit of nAChR, especially when the nucleic acid molecule encoding each individual nAChR subunit is disclosed herein. In view of the above data, it is not seen why the proposed combinations appearing below would not act in a manner similar to the multimeric subunit combination discussed immediately above.

α2β4α6

α3β4α6

α4β4α5

α4β4α6

α4β2α5

α4β2β3

α3β2α6β3

α2β4α5

α2β2α5

αXβ2β4, where X refers to one or more of the α subunits disclosed herein.

αXβ2β3β4, where X refers to one or more of the α subunits disclosed herein

αXb2b3, where X refers to one or more of the α subunits disclosed herein

α2β2α6

α3β2α6

α4β2α6

Five-way combinations of subunits, represented by the general formula α<sub>n</sub>β<sub>m</sub>, wherein n and m are each 0–5 (where

the a subunit is one or more of α<sub>1</sub> thru α<sub>7</sub> and β is any one or more of β<sub>2</sub>, β<sub>3</sub> or β<sub>4</sub> are also contemplated by the present invention. Likewise, four-way combinations are also a feature of the invention.

### 5 III. Recombinant Expression of the Human α7 nAChR in a Non-human Cell Line

#### A. Construct for Expression of Recombinant Human nAChR α<sub>7</sub> in a Non-human Host Cell Line

The isolated cDNA Encoding human neuronal α7 AChR was incorporated into the expression vector pcDNA3 (Invitrogen) for use in expressing the α7 subunit in the GH<sub>4</sub>C<sub>1</sub> host cell line. The expression vector, pcDNA3-KEα7RBS was constructed as described below.

Construct pCMV-KEα7 was digested with BamHI and XhoI to release a 1.8 kb DNA fragment containing a consensus ribosome binding site (RBS) immediately 5' to the translation initiation codon of α7 and also containing the entire α7 coding region. pGEM/KEα7RBS was prepared by ligating this 1.8 kb DNA fragment into BamHI, XhoI digested pGEM-7Zf(+), (Promega). pGEM/KEα7RBS was digested with BamHI and XhoI to release the 1.8 kb DNA fragment containing the RBS and α7 coding region. pcDNA3-KEα7RBS was prepared by ligating the 1.8 kb fragment from pGEM/KEα7RBS into BamHI and XhoI digested pcDNA3.

#### 25 B. Recombinant Expression of the Human α7 nAChR in GH<sub>4</sub>C<sub>1</sub> cells.

GH<sub>4</sub>C<sub>1</sub> cells, derived from rat pituitary tumor tissue, were stably transfected with DNA encoding human neuronal nAChR α7 and analyzed for expression of nAChRs using various assays, for example calcium sensitive fluorescent indicator-based assays, [<sup>125</sup>I] bungarotoxin binding and electrophysiological methods.

##### 1. Stable Transfection of GH<sub>4</sub>C<sub>1</sub> Cells with the Human α7 nAChR.

GH<sub>4</sub>C<sub>1</sub> cells were stably transfected with DNA encoding human neuronal nAChR α7 using the lipofection transfection procedure (Current Protocols in Molecular Biology, Volume 1, 9.4.1–9.4.5 and 9.5.1–9.5.6, incorporated herein by reference).

GH<sub>4</sub>C<sub>1</sub> cells were harvested using Cell Dissociation Buffer (Sigma) and plated onto 10 cm tissue culture plates coated with poly-d-lysine at a concentration of 1.2 million cells per plate, 24 hours prior to transfection. Six micrograms of the α7 expression vector, pcDNA3-KEα7RBS were diluted in 300 μl of Dulbecco's Modified Eagle Medium (DMEM) and combined with 20 μl of LipofectAMINE™ Reagent (Gibco-BRL) for 15 minutes. The GH<sub>4</sub>C<sub>1</sub> cells were washed twice with DMEM. This DNA/LipofectAMINE mixture was further diluted into 5.3 ml of DMEM and overlaid onto the GH<sub>4</sub>C<sub>1</sub> cells. The overlaid cells were incubated for 5 hours in an incubator at 37° C., in a humidified atmosphere containing 6% carbon dioxide. Cell plates were washed twice with 5 mls of Ham's F-10 nutrient mixture (GibcoBRL) containing 10% fetal bovine serum, 100 units per ml of penicillin and 100 μg/ml streptomycin then overlaid with 10 ml of complete medium and placed in an incubator for 48 hours.

Forty-eight hours post-transfection, cell plates were split at a 1:4 ratio, generating four culture plates. Twenty hours later, complete medium containing 500 μg/ml of G418 was added to the cells for 14 days. Medium was replaced every 2 to 4 days. After this period, colonies had formed on the plates and were isolated using trypsin-soaked circles of sterile filter paper. 24 isolates were cultured, 18 survived and were expanded for functional assay using fluorescence-based measurements of internal calcium concentrations as described in Example 4 above.

Clones were also screened in a radioligand binding assay using [<sup>125</sup>I]-bungarotoxin. See example 4. Electrophysiological recordings (similar to the procedure outlined in Example 4) also demonstrated currents with biophysical properties characteristic of the  $\alpha 7$  receptor. Parental cell line G1-9 exhibited robust expression in both functional calcium and electrophysiological assays and in binding assays. The G1-9 parental cell line was subcloned by limiting dilution.

Twenty eight subclones from G1-9 were screened in the fluorescence-based calcium assay. Ten subclones were positive in this assay and showed epibatidine-induced increases in internal calcium. An additional binding assay, similar to that outlined above, identified thirteen positive subclones.

Five subclones, including subclone G1-19-15 were selected based on activity in both calcium and binding assays.

G1-9-15 was further subcloned by limiting dilution, subclones were screened for epibatidine-induced increases in internal calcium. Four subclones, G1-9-15-8, G1-9-15-18, G1-9-15-28 and G1-9-15-35 were selected based on a positive functional response in this assay. These subclones then entered a stability study where they were monitored for functional response in the calcium assay at two-weekly intervals for approximately 15 weeks.

Subclone G1-9-15-8 was selected based on the stable functional response observed during the stability study. This cell line was confirmed to have acceptable responses as a random screening target in the high throughput screening assay and renamed A7 after validation in this assay.

## 2. Analysis of Transfectants

### a. Fluorescence-based Measurements of Internal Calcium Concentrations.

$\text{GH}_4\text{C}_1$  cells that were stably transfected with DNA encoding the human  $\alpha 7$  subunit were analyzed for expression of functional recombinant nAChRs using the automated fluorescent indicator-based assay.

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including  $\text{Ca}^{++}$ , through the receptor channel.  $\text{Ca}^{++}$  entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic  $\text{Ca}^{++}$  levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular  $\text{Ca}^{2+}$  concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying nicotinic AChR has been described in commonly assigned pending U.S. patent application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

Untransfected  $\text{GH}_4\text{C}_1$  cells and  $\text{GH}_4\text{C}_1$  cells that had been transfected with pcDNA3-KE $\alpha 7$ RBS were plated in the wells of a poly-D-lysine coated 96-well microtiter dish at a

cell density of 75,000 to 200,000 cells per well. Twenty four hours after plating, cell culture medium was decanted and cells washed with an assay buffer (HBK) containing 155 mM NaCl, 4.6 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 1.8 mM  $\text{CaCl}_2$ , 1  $\mu\text{M}$  atropine, 6 mM glucose and 20 mM Hepes-NaOH pH7.4. Washed cells were incubated with 20  $\mu\text{M}$  fluo-3-acetoxymethyl ester containing 0.16% pluronic F-127 at 22° C. for 2 hours in the dark. Dye not taken up by cells was removed by aspiration followed by washing with 250  $\mu\text{l}$  HBK. Fluorescence measurements were performed at 0.33 sec intervals using a 96-well microtiter plate-reading fluorometer (Cambridge Technology, Inc.). Cells were incubated for 10 minutes with 3  $\mu\text{M}$  FPL 64176 and ten basal fluorescence readings were recorded prior to addition of 1  $\mu\text{M}$  epibatidine. Responses after the addition of epibatidine were recorded for approximately 60 sec. Alpha-bungarotoxin was tested after a preincubation period of 5–10 min. Maximal fluorescence ( $F_{max}$ ) was determined after lysing the cells with 0.25% Triton X-100, and minimal fluorescence ( $F_{min}$ ) was determined after subsequent quenching with 10 mM  $\text{MnCl}_2$ . Calculation of  $[\text{Ca}^{2+}]_i$  was performed as described by Kao et al. (1989). Cellular responses were quantitated by calculating either the ratio of peak  $[\text{Ca}^{2+}]_i$  after agonist addition to the basal  $[\text{Ca}^{2+}]_i$  prior to agonist addition, or by the difference between peak  $[\text{Ca}^{2+}]_i$  and basal  $[\text{Ca}^{2+}]_i$ .

### b. $\alpha$ -Bungarotoxin Binding Assays

Untransfected  $\text{GH}_4\text{C}_1$  cells and  $\text{GH}_4\text{C}_1$  cells that were stably transfected with DNA encoding the human  $\alpha 7$  subunit were analyzed for [<sup>125</sup>I]- $\alpha$ -bungarotoxin binding. The assay procedure was as follows.

Cells were incubated with 1 nM [<sup>125</sup>I]- $\alpha$  BTX in culture media for 2 hours at room temperature. Non-specific binding was determined in the presence of 1  $\mu\text{M}$  unlabeled toxin. The assays were terminated by aspiration of the culture media and rapid filtration through Whatman GF/C filters using a Brandel Cell Harvester. Filters were washed with approximately 4x1 ml washes of ice cold binding assay buffer (50 mM tris, 140 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , pH 7.4). Filter disks were transferred to scintillation vials containing 5 ml Ecolume scintillation cocktail and radioactivity counted using a Beckman 6500 scintillation spectrometer.

### c. Electrophysiological Analysis of $\text{GH}_4\text{C}_1$ Cells Transfected with Human Neuronal Nicotinic AChR Subunit-encoding DNA (Human $\alpha 7$ Subunit)

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques.

$\text{GH}_4\text{C}_1$  cells stably transfected with DNA encoding the human  $\alpha 7$  subunit were analyzed electrophysiologically for the presence of nAChR agonist-dependent currents.  $\text{GH}_4\text{C}_1$  cells stably expressing human  $\alpha 7$  nAChRs were plated at a density of  $1.5 \times 10^5$  cells/35-mm dish on collagen-coated glass coverslips (rat collagen I, Becton Dickinson) treated with an additional coating of poly-D-lysine (0.1 mg/ml, SIGMA). Recordings were performed with an Axopatch

200A amplifier (Axon Instruments) using the whole-cell voltage-clamp configuration. Membrane potential was held at  $-100$  mV. The standard external recording solution (mammalian Ringer's) consisted of (in mM) 160 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 11 glucose, 0.001 atropine, and 5 HEPES, pH 7.3. Ringer's solution was superfused at a rate of  $\approx 3.0$  ml/min (110  $\mu$ l recording chamber). The recording pipette solution was composed of 135 mM CsCl, 10 mM EGTA, 1 mM MgCl<sub>2</sub> and 10 mM HEPES, pH 7.3 (with or without 4 mM Mg-ATP). Experiments were performed at room temperature. Nicotine (100–300  $\mu$ M), dissolved in Ringer's solution, was applied for 200–500 ms using a fast application system, consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezoelectric drive, Winston Electronics). The speed of solution exchange between control and nicotine-containing solutions, measured as the open-tip response, displays a time constant  $\tau=0.7$  ms, with steady state reached  $<3$  ms. Data were digitized at 6.7 kHz and filtered at 2 kHz on line. Data analysis was performed using pClamp software (Axon Instruments).

#### EXAMPLE 5

##### Characterization of Cell Lines Expressing nNACHRs

Recombinant cell lines generated by transfection with DNA encoding human neuronal nicotinic AChRs, such as those described in Example 3 can be further characterized using one or more of the following methods.

##### A. Northern or Slot Blot Analysis for Expression of $\alpha$ - and/or $\beta$ -subunit Encoding Messages

Total RNA is isolated from  $\sim 1 \times 10^7$  cells and 10–15  $\mu$ g of RNA from each cell type is used for northern or slot blot hybridization analysis. The inserts from human neuronal NACHR-encoding plasmids can be nick-translated and used as probe. In addition, the  $\beta$ -actin gene sequence (Cleveland et al. (1980) Cell 20:95–105) can be nick-translated and used as a control probe on duplicate filters to confirm the presence or absence of RNA on each blot and to provide a rough standard for use in quantitating differences in  $\alpha$ - or  $\beta$ -specific mRNA levels between cell lines. Typical northern and slot blot hybridization and wash conditions are as follows:

hybridization in 5 $\times$ SSPE, 5 $\times$  Denhardt's solution, 50% formamide, at 42 $^{\circ}$  C. followed by washing in 0.2 $\times$  SSPE, 0.1% SDS, at 65 $^{\circ}$  C.

##### B. Nicotine-binding Assay

Cell lines generated by transfection with human neuronal nicotinic AChR  $\alpha$ - or  $\alpha$ - and  $\beta$ -subunit-encoding DNA can be analyzed for their ability to bind nicotine, for example, as compared to control cell lines: neuronally-derived cell lines PC12 (Boulter et al., (1986), supra; ATCC #CRL1721) and IMR32 (Clementi, et al. (1986); Int. J. Neurochem. 47:291–297; ATCC #CCL127), and muscle-derived cell line BC3H1 (Patrick, et al, (1977); J. Biol. Chem. 252:2143–2153. Negative control cells (i.e., host cells from which the transfectants were prepared) are also included in the assay. The assay is conducted as follows:

Just prior to being assayed, transfected cells are removed from plates by scraping. Positive control cells used are PC12, BC3H1, and IMR32 (which had been starved for fresh media for seven days). Control cell lines are removed by rinsing in 37 $^{\circ}$  C. assay buffer (50 mM Tris/HCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 120 mM NaCl 3 mM EDTA, 2 mg/ml BSA and 0.1% aprotinin at pH7.4). The cells are washed and resuspended to a concentration of  $1 \times 10^6/250$   $\mu$ l. To each

plastic assay tube is added 250  $\mu$ l of the cell solution, 15 nM <sup>3</sup>H-nicotine, with or without 1 mM unlabeled nicotine, and assay buffer to make a final volume of 500  $\mu$ l. The assays for the transfected cell lines are incubated for 30 min at room temperature; the assays of the positive control cells are incubated for 2 min at 1 $^{\circ}$  C. After the appropriate incubation time, 450  $\mu$ l aliquots of assay volume are filtered through Whatman GF/C glass fiber filters which has been pretreated by incubation in 0.05% polyethyleneimine for 24 hours at 4 $^{\circ}$  C. The filters are then washed twice, with 4 ml each wash, with ice cold assay buffer. After washing, the filters are dried, added to vials containing 5 ml scintillation fluid and radioactivity is measured.

##### C. <sup>86</sup>Rb ion-flux Assay

The ability of nicotine or nicotine agonists and antagonists to mediate the influx of <sup>86</sup>Rb into transfected and control cells has been found to provide an indication of the presence of functional AChRs on the cell surface. The <sup>86</sup>Rb ion-flux assay is conducted as follows:

1. The night before the experiment, cells are plated at  $2 \times 10^6$  per well (i.e., 2 ml per well) in a 6-well polylysine-coated plate.
2. The culture medium is decanted and the plate washed with 2 ml of assay buffer (50 mM HEPES, 260 mM sucrose, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 5.5 mM glucose) at room temperature.
3. The assay buffer is decanted and 1 ml of assay buffer, containing 3  $\mu$ Ci/ml <sup>86</sup>Rb, with 5 mM ouabain and agonist or antagonist in a concentration to effect a maximum response, is added.
4. The plate is incubated on ice at 1 $^{\circ}$  C. for 4 min.
5. The buffer is decanted into a waste container and each well was washed with 3 ml of assay buffer, followed by two washes of 2 ml each.
6. The cells are lysed with 2 $\times$ 0.5 ml of 0.2% SDS per well and transferred to a scintillation vial containing 5 ml of scintillation fluid.
7. The radioactivity contained in each vial is measured and the data calculated.

Positive control cells provided the following data in this assay:

	PC12		IMR32	
	EC <sub>50</sub>	Maximum response	EC <sub>50</sub>	Maximum response
<u>Agonist</u>				
nicotine	52 $\mu$ M	2.1 X <sup>a</sup>	18 $\mu$ M	7.7 X <sup>a</sup>
CCh*	35 $\mu$ M	3.3 X <sup>b</sup>	230 $\mu$ M	7.6 X <sup>c</sup>
cytisine	57 $\mu$ M	3.6 X <sup>d</sup>	14 $\mu$ M	10 X <sup>e</sup>
<u>Antagonist</u>				
d-tubocurarine	0.81 $\mu$ M		2.5 $\mu$ M	
mecamylamine	0.42 $\mu$ M		0.11 $\mu$ M	
hexamethonium		nd <sup>f</sup>	22 $\mu$ M	
atropine	12.5 $\mu$ M		43 $\mu$ M	

\*CCh = carbamylcholine

<sup>a</sup>200  $\mu$ M nicotine

<sup>b</sup>300  $\mu$ M CCh

<sup>c</sup>3 mM CCh

<sup>d</sup>1 mM cytisine

<sup>e</sup>100  $\mu$ M cytisine

<sup>f</sup>nd = not determined

##### D. Electrophysiological Analysis of Mammalian Cells Transfected with Human Neuronal Nicotinic AChR Subunit-encoding DNA

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability

of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques. In preferred embodiments, transfected mammalian cells or injected oocytes are analyzed electrophysiologically for the presence of AChR agonist-dependent currents.

#### EXAMPLE 6

##### Characterization of GH<sub>4</sub>C<sub>1</sub> Cells Stably Expressing the Human $\alpha$ 7 nAChR

The cell line A7 that stably expressed the human  $\alpha$ 7 nAChR was characterized in multiple assays that are described below.

Dose response curves to reference nicotinic agonists nicotine and acetylcholine were obtained for cell line A7 using the fura-2 based calcium assay. See protocol A infra.

Referring to FIG. 3, the EC<sub>50</sub> for nicotine was 2  $\mu$ M and for acetylcholine was 7  $\mu$ M. This is in agreement with that reported for the  $\alpha$ 7 nAChR (Peng et al (1993) Mol Pharmacol. 45:546-554).

Data on electrophysiological characterization using whole-cell voltage-clamped A7 cells is depicted in FIG. 4, which show rapidly desensitizing currents that are consistent with those reported for  $\alpha$ 7 nAChRs. The protocols for these experiments were the same as those described in Examples 3 and 4 above. In these studies 90% to 100% of voltage-clamped A7 cells responded to the application of 300  $\mu$ M nicotine.

Single cell calcium imaging of the A7 cell line (FIG. 6) (protocol B, infra) supports the conclusion that individual cells in this cell line (A7) respond to 10  $\mu$ M epibatidine in a homogenous manner.

In radioligand binding studies (protocol C, infra) methyllycaconitine (MLA) displaced [<sup>3</sup>H]-MLA binding from the  $\alpha$ 7 nAChRs in cell line A7 with an IC<sub>50</sub> of 4 nM, similar to the IC<sub>50</sub> value obtained with  $\alpha$ -bungarotoxin (3 nM). These IC<sub>50</sub> values are similar to published affinities (for example, Davies et al. 1999, Neuropharmacology 38:679).  $\alpha$ -bungarotoxin displaced approximately 65% of the [<sup>3</sup>H]-MLA binding in A7. Cells are permeable to MLA but not to  $\alpha$ -bungarotoxin under these assay conditions. This therefore demonstrates that 65% of the  $\alpha$ 7 nAChRs in cell line A7 are expressed on the plasma membrane (i.e. at the cell surface). This data is illustrated in FIG. 5.

A molecular characterization was undertaken to demonstrate the expression of  $\alpha$ 7 nAChR protein and  $\alpha$ 7 mRNA in the stable cell line A7. Western analysis using an  $\alpha$ 7-specific antibody demonstrated that cell line A7 expressed protein of approximately 54 kDa. Protein prepared from the untransfected GH<sub>4</sub>C<sub>1</sub> cell line does not show any hybridization with this antibody. Refer to FIG. 7.

Northern analysis of total RNA prepared from A7 cells showed that these cells express an RNA species that hybridizes with a subunit specific DNA probe. The hybridizing band has a molecular weight of approximately 2.4 kb. No hybridizing species was detected in untransfected GH<sub>4</sub>C<sub>1</sub> cells. Refer to FIG. 8.

The characterizations of stable cell line A7 described above were generated using the following protocols.

##### A. Fluorescence Based Calcium Assays using Fura-2

A cell line A7 stably transfected with the human  $\alpha$ 7 nAChR receptor is plated in black-walled 96-well plates and grown at 37° C. Twenty-four hours later, the plates are washed with in HEPES buffered saline (HBS) containing 1  $\mu$ M atropine (HBSA) (wash cycle=aspirate, dispense $\times$ 3) to leave 180  $\mu$ l residual HBSA per well. At the start of the assay, a background measurement of a sample plate was taken by the SpeedReader for 20 frames alternating the excitation light between 350 and 385 nm at four hertz. See U.S. Pat. Nos. 5,670,113 and 6,057,114, each of which is incorporated by reference herein in their entirety. Twenty  $\mu$ l of 10  $\mu$ M fura-2 dye containing 3  $\mu$ M FPL-64176 is then added to each well and incubated with the cells at ambient temperature for one to two hours. After dye loading the free dye is washed from the wells with HBSA containing 0.5  $\mu$ M FPL-64176 to leave 180  $\mu$ l residual buffer per well. Two minutes after washing, a kinetic reading is taken while the test chemicals are added. The test compounds are prepared in HBSA containing 80 mM CaCl<sub>2</sub> and 1% DMSO. The kinetic reading is composed of 140 frames, alternating between 350 and 385 as in the 20 frame background reading. However, the first 20 frames of the kinetic reading are taken before test chemical addition. The difference between these 20 frames and the background give the fluorescence due to the calcium-indicating dye fura-2. After the first 20 frames are collected 20  $\mu$ l of the test compound is dispensed from a 96-channel pipettor to the entire plate at once without halting the reading. The remainder of the 120 frames of data measure the response.

Absolute calcium concentrations are not calculated from these readings, rather the directly measured fluorescence ratio is used as a surrogate for calcium. The fluorescence ratio is calculated as dye fluorescence generated by excitation at 350 nm divided by dye fluorescence generated by excitation at 385 nm. The raw activity in a well is calculated as the maximum fluorescence ratio after compound addition divided by the average fluorescence ratio before compound addition.

##### B. Single Cell Calcium Imaging Assays using Fura-2

Cells stably transfected with the human  $\alpha$ 7 nAChR were plated on poly-D-lysine-coated glass coverslips at a density of 3 $\times$ 10<sup>5</sup> cells/35 mm dish. Twenty four hours later, imaging experiments were performed at room temperature, using a Nikon TE200 inverted microscope attached to a DeltaRAM imaging System (Photon Technology International). Cells were incubated with 1  $\mu$ M fura-2-AM (Molecular Probes, Inc.) for 0.5-1 h and washed with mammalian Ringer's solution (see example above re: the ephys composition of this buffer eg Ringers (in mM) 160 NaCl, 5 KCl, 1 MgCl etc.) to remove excess dye. Cells were transferred to a recording chamber (110  $\mu$ l, Warner Instruments), and continuously superfused with HBK containing 1  $\mu$ M atropine at a rate of 8-10 ml/min. 10  $\mu$ M epibatidine was applied by switching between reservoirs. Cells were alternatively excited at 360 and 381 nm (0.5 Hz) to determine ratio images.

##### C. Radioligand Binding Studies

GH4C1 cells stably expressing  $\alpha$ 7 were plated in 96-well microtiter plates at a density of 200,000 cells per well. Twenty-four hours later, cells were washed in assay buffer (50 mM Tris, 140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4), and incubated with 1 nM [<sup>3</sup>H]-methyllycaconitine in the presence of increasing concentrations of either methyllycaconitine (MLA) or

$\alpha$ -bungarotoxin. After 120 min, the assay was terminated by aspiration of the buffer and rapid filtration through Whatman GF/C filters using a Brandel Cell Harvester. Filters were washed with approximately 4×1 ml washes of ice cold assay buffer, and filter disks transferred to scintillation vials containing 5 ml Ecolume scintillation cocktail. Radioactivity was counted using a Beckman 6500 scintillation spectrometer. Specific binding was calculated by subtracting the non-specific binding, defined by 10  $\mu$ M MLA.

#### D. Western Analysis for Expression of $\alpha$ 7 Protein

Cells stably transfected with the human  $\alpha$ 7 nAChR were harvested from 10-cm plates and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4). Washed cells were resuspended in 50 mM Tris pH 7.4, 1 mM EDTA containing a cocktail of protease inhibitors (Complete™, Boehringer Mannheim, Indianapolis, Ind.) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000×g for 5 min to remove cellular debris, and the supernatant fraction was centrifuged at 100,000×g for 120 min to pellet the membranes. The membranes were resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1% SDS) containing protease inhibitor cocktail.

For immunoblot analysis, membranes were solubilized in Tris-Glycine SDS Sample Buffer (Novex, San Diego, Calif.) containing 5% 2-mercaptoethanol and heated at 65° C. for 10 min. Solubilized proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hy-Bond ECL, Amersham, Arlington Heights, Ill.). Blots were rinsed once in PBS, 0.1% Tween-20 (wash buffer), then blocked for 3 h in 5% Carnation non-fat dry milk dissolved in wash buffer (blocking buffer).

The human  $\alpha$ 7 protein was detected with an antibody raised in goat against a human  $\alpha$ 7-specific peptide (Santa Cruz Biotechnology). The primary antibody was diluted to 0.5  $\mu$ g/ml in blocking buffer and incubated with the nitrocellulose membrane for 3 h at room temperature. The membranes were washed three times in wash buffer. The secondary antibody was peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) diluted 1:2500 in blocking buffer and incubated with membranes for 45 min at room temperature, followed by five changes of wash buffer. The antibody signal was visualized using the ECL developing system (Amersham) according to the manufacturer's directions.

#### E. Northern Analysis for Expression of $\alpha$ 7 Encoding Message.

Total RNA was isolated from approximately  $1 \times 10^7$  cells for northern hybridization analysis. Total RNA was size-fractionated on an agarose-formaldehyde gel and blotted to nylon by downward alkaline transfer. Blots were hybridized with digoxigenin-labeled DNA probes specific for human  $\alpha$ 7 subunits (nucleic acid numbers 1066–1533). Blots were hybridized overnight with 20 ng/ml probe and washed at high stringency in a wash buffer containing 0.1×SSPE (3 mM NaCl, 0.2 mM  $\text{NaH}_2\text{PO}_4$ , 0.02 mM EDTA) and 0.1% SDS at 65° C. Chemiluminescent detection was performed using the Genius 7 kit (Boehringer Mannheim) according to the manufacturer's instructions. Refer to FIG. 8.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

#### SUMMARY OF SEQUENCES

Sequence ID No. 1 is a nucleotide sequence encoding an  $\alpha_2$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 2 is the amino acid sequence of the  $\alpha_2$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 1.

Sequence ID No. 3 is a nucleotide sequence encoding an  $\alpha_3$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 4 is the amino acid sequence of the  $\alpha_3$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 3.

Sequence ID No. 5 is a nucleotide sequence encoding an  $\alpha_4$  subunit of a human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 6 is the amino acid sequence of the  $\alpha_4$  subunit of a human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 5.

Sequence ID No. 7 is a nucleotide sequence encoding an  $\alpha_5$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 8 is the amino acid sequence of the  $\alpha_5$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 7.

Sequence ID No. 9 is a nucleotide sequence encoding an  $\alpha_6$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 10 is the amino acid sequence of the  $\alpha_6$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 9.

Sequence ID No. 11 is a nucleotide sequence encoding an  $\alpha_7$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 12 is the amino acid sequence of the  $\alpha_7$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 11.

Sequence ID No. 13 is a nucleotide sequence encoding a  $\beta_2$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 14 is the amino acid sequence of the  $\beta_2$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 13.

Sequence ID No. 15 is a nucleotide sequence encoding  $\beta_3$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 16 is the amino acid sequence of the  $\beta_3$  subunit of human neuronal nicotinic acetylcholine receptor, set forth in Sequence ID No. 15.

Sequence ID No. 17 is a nucleotide sequence encoding a  $\beta_4$  subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 18 is the amino acid sequence of the  $\beta_4$  subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 17.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 18

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2277 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 166..1755
- (D) OTHER INFORMATION: /product= "ALPHA-2 SUBUNIT"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GTTCTCTACA ACAATGCAGA TGGGGAGTTT GCAGTGACCC ACATGACCAA GGCCACCTC    660
TTCTCCACGG GCACTGTGCA CTGGGTGCC CCGGCCATCT ACAAGAGCTC CTGCAGCATC    720
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TATGACAAGG CCAAGATCGA CCTGGAGCAG ATGGAGCAGA CTGTGGACCT GAAGGACTAC    840
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CTCTTCTACA CCATCAACCT CATCATCCCC TGCCTGCTCA TCTCCTGCCT CACTGTGCTG   1020
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GCAGGTCATG TGGCCCCCTC TGTGGGCACC CTCTGCAGCC ACGGCCACCT GCACTCTGGG   1500
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AGTAATAAGG GCTCTTCCGG AAGGGGAGGA AGCGGGAGGC AGGGCCTGCA CCTGATGTGG 2040  
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 529 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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20 25 30  
Pro Pro Arg Ala Pro Gly Asp Pro Leu Ser Ser Pro Ser Pro Thr Ala  
35 40 45  
Leu Pro Gln Gly Gly Ser His Thr Glu Thr Glu Asp Arg Leu Phe Lys  
50 55 60  
His Leu Phe Arg Gly Tyr Asn Arg Trp Ala Arg Pro Val Pro Asn Thr  
65 70 75 80  
Ser Asp Val Val Ile Val Arg Phe Gly Leu Ser Ile Ala Gln Leu Ile  
85 90 95  
Asp Val Asp Glu Lys Asn Gln Met Met Thr Thr Asn Val Trp Leu Lys  
100 105 110  
Gln Glu Trp Ser Asp Tyr Lys Leu Arg Trp Asn Pro Ala Asp Phe Gly  
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165 170 175  
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180 185 190  
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195 200 205  
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			260					265					270		
Ile	Ile	Pro	Cys	Leu	Leu	Ile	Ser	Cys	Leu	Thr	Val	Leu	Val	Phe	Tyr
			275					280					285		
Leu	Pro	Ser	Asp	Cys	Gly	Glu	Lys	Ile	Thr	Leu	Cys	Ile	Ser	Val	Leu
	290						295				300				
Leu	Ser	Leu	Thr	Val	Phe	Leu	Leu	Leu	Ile	Thr	Glu	Ile	Ile	Pro	Ser
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Tyr	His	Trp	Leu	Glu	Ser	Asn	Val	Asp	Ala	Glu	Glu	Arg	Glu	Val	Val
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Val	Glu	Glu	Glu	Asp	Arg	Trp	Ala	Cys	Ala	Gly	His	Val	Ala	Pro	Ser
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Ile

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1654 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 39..1553
- (D) OTHER INFORMATION: /product= "ALPHA-3 SUBUNIT"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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 GGATAAAAAT CTCCAATTTT AGTGCTAACC TCACGAGAAG CTCTAGTTCT GAATCTGTTG 1320  
 ATGCTGTGCT GTCCCTCTCT GCTTTGTGAC CAGAAATCAA AGAAGCCATC CAAAGTGTC 1380  
 AGTATATTGC TGAAAATATG AAAGCACAAA ATGAAGCCAA AGAGATTCAA GATGATTGGA 1440  
 AGTATGTTGC CATGGTGATT GATCGTATTT TTCTGTGGGT TTTCACCCTG GTGTGCATTC 1500  
 TAGGGACAGC AGGATTGTTT CTGCAACCCC TGATGGCCAG GGAAGATGCA TAAGCACTAA 1560  
 GCTGTGTGCC TGCCCTGGGAG ACTTCCTTGT GTCAGGGCAG GAGGAGGCTG CTTCTAGTA 1620  
 AGAACGTACT TTCTGTTATC AAGCTACCAG CTTT 1654

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 504 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Gly Ser Gly Pro Leu Ser Leu Pro Leu Ala Leu Ser Pro Pro Arg  
 1 5 10 15  
 Leu Leu Leu Leu Leu Ser Leu Leu Pro Val Ala Arg Ala Ser Glu  
 20 25 30  
 Ala Glu His Arg Leu Phe Glu Arg Leu Phe Glu Asp Tyr Asn Glu Ile  
 35 40 45  
 Ile Arg Pro Val Ala Asn Val Ser Asp Pro Val Ile Ile His Phe Glu  
 50 55 60  
 Val Ser Met Ser Gln Leu Val Lys Val Asp Glu Val Asn Gln Ile Met  
 65 70 75 80  
 Glu Thr Asn Leu Trp Leu Lys Gln Ile Trp Asn Asp Tyr Lys Leu Lys



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## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2363 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 173..2056
- (D) OTHER INFORMATION: /product= "ALPHA-4 SUBUNIT"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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GCGCTCGCTG CGGCGCCGCC GCCGCNCCGC GCGCCACAGG AGAAGGCGAN CCGGGCCCGG      60
CGGCCGAAGC GGCCCGCGAG GCGCGGGAGG CATGAAGTTG GCGCGGCACG GGCCTCGAAG      120
CGGCGGGGAG CCGGGAGCCG CCCGCATCTA GAGCCCAGCA GGTGCGTGCG CCATGGAGCT      180
AGGGGGCCCC GGAGCGCCGC GGCTGCTGCC GCCGCTGCTG CTGCTTCTGG GGACCGGCCT      240
CCTGCGCGCC AGCAGCCATG TGGAGACCCG GGCCACGCC GAGGAGCGGC TCCTGAAGAA      300
ACTCTTCTCC GGTTACAACA AGTGGTCCCG ACCCGTGGCC AACATCTCGG ACGTGGTCTT      360
CGTCCGCTTC GGCCTGTCCA TCGCTCAGCT CATTGACGTG GATGAGAAGA ACCAGATGAT      420
GACCACGAAC GTCTGGGTGA AGCAGGAGTG GCACGACTAC AAGCTGCGCT GGGACCCAGC      480
TGACTIONGAG AATGTCACCT CCATCCGCAT CCCCTCCGAG CTCATCTGGC GGCCGGACAT      540
CGCCCTCTAC AACAAATGCTG ACGGGGACTT CGCGGCCACC CACCTGACCA AGGCCACCT      600
GTTCCATGAC GGGCGGGTGC AGCGGACTCC CCCGGCCATT TACAAGAGCT CCTGCAGCAT      660
CGACGTCACC TTCTTCCCCT TCGACCAGCA GAACTGCACC ATGAAATTCG GCTCCTGGAC      720
CTACGACAAG GCCAAGATCG ACCTGGTGAA CATGCACAGC CGCGTGGACC AGCTGGACTT      780
CTGGGAGAGT GCGGAGTGGC TCATCTCGGA CGCCGTGGGC ACCTACAACA CCAGGAAGTA      840
CGAGTGCTGC GCCGAGATCT ACCCGGACAT CACCTATGCC TACGCCATCC GCGGGCTGCC      900
GCTCTTCTAC ACCATCAACC TCATCATCCC CTGGCTGCTC ATCTCCTGCC TCACCGCGCT      960
GGTCTTCTAC CTGCCCTCCG AGTGTGGCGA GAAGATCAGC CTGTGCATCT CCGTGCTGCT     1020
GTCGCTCACC GTCTTCCTGC TGCTCATCAC CGAGATCATC CCGTCCACCT CACTGGTCAT     1080
CCCCTCATC GCGGAGTACC TGCTGTTTAC CATGATCTTC GTCACCCTGT CCATCGCCAT     1140
CACGGTCTTC GTGCTCAACG TGCACCACCG CTCGCCACGC ACGCACACCA TGCCACCTG     1200
GGTACGCAGG GTCTTCCTGG ACATCGTGCC ACGCCTGCTC CTCATGAAGC GGCCGTCCGT     1260
GGTCAAGGAC AATTGCCGGC GGCTCATCGA GTCCATGCAT AAGATGGCCA GTGCCCCGCG     1320
CTTCTGGCCC GAGCCAGAAG GGGAGCCCCC TGCCACGAGC GGCACCCAGA GCCTGCACCC     1380
TCCCTCACCG TCCTTCTGCG TCCCCCTGGA TGTGCCGGCT GAGCCTGGGC CTTCCTGCAA     1440
GTCACCCTCC GACCAGCTCC CTCCTCAGCA GCCCCTGGAA GCTGAGAAAG CCAGCCCCCA     1500
CCCCTCGCCT GGACCCTGCC GCCC GCCCA CGGCACCCAG GCACCAGGC TGGCCAAAGC     1560
CAGGTCCCTC AGCGTCCAGC ACATGTCCAG CCCTGGCGAA GCGGTGGAAG GCGGCGTCCG     1620
GTGCCGGTCT CGGAGCATCC AGTACTGTGT TCCCCGAGAC GATGCCGCC CCGAGGCAGA     1680
TGGCCAGGCT GCCGGCGCCC TGGCCTCTCG CAACAGCCAC TCGGCTGAGC TCCCACCCCC     1740
AGACCAGCCC TCTCCGTGCA AATGCACATG CAAGAAGGAG CCCTCTTCGG TGTCCCCGAG     1800

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CGCCACGGTC AAGACCCGCA GCACCAAAGC GCCGCCCGG CACCTGCCCC TGTCGCCGGC 1860  
 CCTGAGCCGG GCGGTGGAGG GCGTCCAGTA CATTGCAGAC CACCTGAAGG CCGAAGACAC 1920  
 AGACTTCTCG GTGAAGGAGG ACTGGAAGTA CGTGGCCATG GTCATCGACC GCATCTTCCT 1980  
 CTGGATGTTT ATCATCGTCT GCCTGCTGGG GACGGTGGGC CTCTTCCTGC CGCCCTGGCT 2040  
 GGCTGGCATG ATCTAGGAAG GGACCGGGAG CCTGCGTGGC CTGGGGCTGC CGYGCACGGG 2100  
 GCCAGCATCC ATGCGGCCCG CCTGGGGCCG GGCTGGCTTC TCCCTGGACT CTGTGGGGCC 2160  
 ACACGTTTGC CAAATTTTCC TTCCTGTTCT GTGTCTGCTG TAAGACGGCC TTGGACGGCG 2220  
 ACACGGCCTC TGGGGAGACC GAGTGTGGAG CTGCTTCCAG TTGGACTCTS GCCTCAGNAG 2280  
 GCAGCGGCTT GGAGCAGAGG TGGCGGTCGC CGCCTYCTAC CTGCAGGACT CGGGCTAAGT 2340  
 CCAGCTCTCC CCCTGCGCAG CCC 2363

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 627 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Glu Leu Gly Gly Pro Gly Ala Pro Arg Leu Leu Pro Pro Leu Leu  
 1 5 10 15  
 Leu Leu Leu Gly Thr Gly Leu Leu Arg Ala Ser Ser His Val Glu Thr  
 20 25 30  
 Arg Ala His Ala Glu Glu Arg Leu Leu Lys Lys Leu Phe Ser Gly Tyr  
 35 40 45  
 Asn Lys Trp Ser Arg Pro Val Ala Asn Ile Ser Asp Val Val Leu Val  
 50 55 60  
 Arg Phe Gly Leu Ser Ile Ala Gln Leu Ile Asp Val Asp Glu Lys Asn  
 65 70 75 80  
 Gln Met Met Thr Thr Asn Val Trp Val Lys Gln Glu Trp His Asp Tyr  
 85 90 95  
 Lys Leu Arg Trp Asp Pro Ala Asp Tyr Glu Asn Val Thr Ser Ile Arg  
 100 105 110  
 Ile Pro Ser Glu Leu Ile Trp Arg Pro Asp Ile Ala Leu Tyr Asn Asn  
 115 120 125  
 Ala Asp Gly Asp Phe Ala Ala Thr His Leu Thr Lys Ala His Leu Phe  
 130 135 140  
 His Asp Gly Arg Val Gln Arg Thr Pro Pro Ala Ile Tyr Lys Ser Ser  
 145 150 155 160  
 Cys Ser Ile Asp Val Thr Phe Phe Pro Phe Asp Gln Gln Asn Cys Thr  
 165 170 175  
 Met Lys Phe Gly Ser Trp Thr Tyr Asp Lys Ala Lys Ile Asp Leu Val  
 180 185 190  
 Asn Met His Ser Arg Val Asp Gln Leu Asp Phe Trp Glu Ser Gly Glu  
 195 200 205  
 Trp Leu Ile Ser Asp Ala Val Gly Thr Tyr Asn Thr Arg Lys Tyr Glu  
 210 215 220  
 Cys Cys Ala Glu Ile Tyr Pro Asp Ile Thr Tyr Ala Tyr Ala Ile Arg  
 225 230 235 240  
 Arg Leu Pro Leu Phe Tyr Thr Ile Asn Leu Ile Ile Pro Trp Leu Leu  
 245 250 255

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Ile Ser Cys Leu Thr Ala Leu Val Phe Tyr Leu Pro Ser Glu Cys Gly  
 260 265 270  
 Glu Lys Ile Thr Leu Cys Ile Ser Val Leu Leu Ser Leu Thr Val Phe  
 275 280 285  
 Leu Leu Leu Ile Thr Glu Ile Ile Pro Ser Thr Ser Leu Val Ile Pro  
 290 295 300  
 Leu Ile Gly Glu Tyr Leu Leu Phe Thr Met Ile Phe Val Thr Leu Ser  
 305 310 315 320  
 Ile Ala Ile Thr Val Phe Val Leu Asn Val His His Arg Ser Pro Arg  
 325 330 335  
 Thr His Thr Met Pro Thr Trp Val Arg Arg Val Phe Leu Asp Ile Val  
 340 345 350  
 Pro Arg Leu Leu Leu Met Lys Arg Pro Ser Val Val Lys Asp Asn Cys  
 355 360 365  
 Arg Arg Leu Ile Glu Ser Met His Lys Met Ala Ser Ala Pro Arg Phe  
 370 375 380  
 Trp Pro Glu Pro Glu Gly Glu Pro Pro Ala Thr Ser Gly Thr Gln Ser  
 385 390 395 400  
 Leu His Pro Pro Ser Pro Ser Phe Cys Val Pro Leu Asp Val Pro Ala  
 405 410 415  
 Glu Pro Gly Pro Ser Cys Lys Ser Pro Ser Asp Gln Leu Pro Pro Gln  
 420 425 430  
 Gln Pro Leu Glu Ala Glu Lys Ala Ser Pro His Pro Ser Pro Gly Pro  
 435 440 445  
 Cys Arg Pro Pro His Gly Thr Gln Ala Pro Gly Leu Ala Lys Ala Arg  
 450 455 460  
 Ser Leu Ser Val Gln His Met Ser Ser Pro Gly Glu Ala Val Glu Gly  
 465 470 475 480  
 Gly Val Arg Cys Arg Ser Arg Ser Ile Gln Tyr Cys Val Pro Arg Asp  
 485 490 495  
 Asp Ala Ala Pro Glu Ala Asp Gly Gln Ala Ala Gly Ala Leu Ala Ser  
 500 505 510  
 Arg Asn Ser His Ser Ala Glu Leu Pro Pro Pro Asp Gln Pro Ser Pro  
 515 520 525  
 Cys Lys Cys Thr Cys Lys Lys Glu Pro Ser Ser Val Ser Pro Ser Ala  
 530 535 540  
 Thr Val Lys Thr Arg Ser Thr Lys Ala Pro Pro Pro His Leu Pro Leu  
 545 550 555 560  
 Ser Pro Ala Leu Ser Arg Ala Val Glu Gly Val Gln Tyr Ile Ala Asp  
 565 570 575  
 His Leu Lys Ala Glu Asp Thr Asp Phe Ser Val Lys Glu Asp Trp Lys  
 580 585 590  
 Tyr Val Ala Met Val Ile Asp Arg Ile Phe Leu Trp Met Phe Ile Ile  
 595 600 605  
 Val Cys Leu Leu Gly Thr Val Gly Leu Phe Leu Pro Pro Trp Leu Ala  
 610 615 620  
 Gly Met Ile  
 625

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1828 base pairs  
 (B) TYPE: nucleic acid





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AAG CGC CTG CCT CTC TTT TAT ACC TTG TTC CTT ATA ATA CCC TGT ATT	940
Lys Arg Leu Pro Leu Phe Tyr Thr Leu Phe Leu Ile Ile Pro Cys Ile	
250 255 260	
GGG CTC TCA TTT TTA ACT GTA CTT GTC TTC TAT CTT CCT TCA AAT GAA	988
Gly Leu Ser Phe Leu Thr Val Leu Val Phe Tyr Leu Pro Ser Asn Glu	
265 270 275	
GGT GAA AAG ATT TGT CTC TGC ACT TCA GTA CTT GTG TCT TTG ACT GTC	1036
Gly Glu Lys Ile Cys Leu Cys Thr Ser Val Leu Val Ser Leu Thr Val	
280 285 290	
TTC CTT CTG GTT ATT GAA GAG ATC ATA CCA TCA TCT TCA AAA GTC ATA	1084
Phe Leu Leu Val Ile Glu Glu Ile Ile Pro Ser Ser Ser Lys Val Ile	
295 300 305 310	
CCT CTA ATT GGA GAG TAT CTG GTA TTT ACC ATG ATT TTT GTG ACA CTG	1132
Pro Leu Ile Gly Glu Tyr Leu Val Phe Thr Met Ile Phe Val Thr Leu	
315 320 325	
TCA ATT ATG GTA ACC GTC TTC GCT ATC AAC ATT CAT CAT CGT TCT TCC	1180
Ser Ile Met Val Thr Val Phe Ala Ile Asn Ile His His Arg Ser Ser	
330 335 340	
TCA ACA CAT AAT GCC ATG GCG CCT TTG GTC CGC AAG ATA TTT CTT CAC	1228
Ser Thr His Asn Ala Met Ala Pro Leu Val Arg Lys Ile Phe Leu His	
345 350 355	
ACG CTT CCC AAA CTG CTT TGC ATG AGA AGT CAT GTA GAC AGG TAC TTC	1276
Thr Leu Pro Lys Leu Leu Cys Met Arg Ser His Val Asp Arg Tyr Phe	
360 365 370	
ACT CAG AAA GAG GAA ACT GAG AGT GGT AGT GGA CCA AAA TCT TCT AGA	1324
Thr Gln Lys Glu Glu Thr Glu Ser Gly Ser Gly Pro Lys Ser Ser Arg	
375 380 385 390	
AAC ACA TTG GAA GCT GCG CTC AAT TCT ATT CGC TAC ATT ACA AGA CAC	1372
Asn Thr Leu Glu Ala Ala Leu Asn Ser Ile Arg Tyr Ile Thr Arg His	
395 400 405	
ATC ATG AAG GAA AAT GAT GTC CGT GAG GTT GTT GAA GAT TGG AAA TTC	1420
Ile Met Lys Glu Asn Asp Val Arg Glu Val Val Glu Asp Trp Lys Phe	
410 415 420	
ATA GCC CAG GTT CTT GAT CGG ATG TTT CTG TGG ACT TTT CTT TTC GTT	1468
Ile Ala Gln Val Leu Asp Arg Met Phe Leu Trp Thr Phe Leu Phe Val	
425 430 435	
TCA ATT GTT GGA TCT CTT GGG CTT TTT GTT CCT GTT ATT TAT AAA TGG	1516
Ser Ile Val Gly Ser Leu Gly Leu Phe Val Pro Val Ile Tyr Lys Trp	
440 445 450	
GCA AAT ATA TTA ATA CCA GTT CAT ATT GGA AAT GCA AAT AAG TGAAGCCTCC	1568
Ala Asn Ile Leu Ile Pro Val His Ile Gly Asn Ala Asn Lys	
455 460 465	
CAAGGGACTG AAGTATACAT TTAGTTAACA CACATATATC TGATGGCACC TATAAAATTA	1628
TGAAAATGTA AGTTATGTGT TAAATTTAGT GCAAGCTTTA ACAGACTAAG TTGCTAACCT	1688
CAATTTATGT TAACAGATGA TCCATTTGAA CAGTTGGCTG TATGACTGAA GTAATAACTG	1748
ATGAGATACA TTTGATCTTG TAAAAATAGC AAAATATTAT CTGAACTGGA CTAGTGAAAA	1808
ATCTAGTATT TGTATCCTGG	1828

## (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 468 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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Met	Ala	Ala	Arg	Gly	Ser	Gly	Pro	Arg	Ala	Leu	Arg	Leu	Leu	Leu	Leu	1	5	10	15
Val	Gln	Leu	Val	Ala	Gly	Arg	Cys	Gly	Leu	Ala	Gly	Ala	Ala	Gly	Gly	20	25	30	
Ala	Gln	Arg	Gly	Leu	Ser	Glu	Pro	Ser	Ser	Ile	Ala	Lys	His	Glu	Asp	35	40	45	
Ser	Leu	Leu	Lys	Asp	Leu	Phe	Gln	Asp	Tyr	Glu	Arg	Trp	Val	Arg	Pro	50	55	60	
Val	Glu	His	Leu	Asn	Asp	Lys	Ile	Lys	Ile	Lys	Phe	Gly	Leu	Ala	Ile	65	70	75	80
Ser	Gln	Leu	Val	Asp	Val	Asp	Glu	Lys	Asn	Gln	Leu	Met	Thr	Thr	Asn	85	90	95	
Val	Trp	Leu	Lys	Gln	Glu	Trp	Ile	Asp	Val	Lys	Leu	Arg	Trp	Asn	Pro	100	105	110	
Asp	Asp	Tyr	Gly	Gly	Ile	Lys	Val	Ile	Arg	Val	Pro	Ser	Asp	Ser	Val	115	120	125	
Trp	Thr	Pro	Asp	Ile	Val	Leu	Phe	Asp	Asn	Ala	Asp	Gly	Arg	Phe	Glu	130	135	140	
Gly	Thr	Ser	Thr	Lys	Thr	Val	Ile	Arg	Tyr	Asn	Gly	Thr	Val	Thr	Trp	145	150	155	160
Thr	Pro	Pro	Ala	Asn	Tyr	Lys	Ser	Ser	Cys	Thr	Ile	Asp	Val	Thr	Phe	165	170	175	
Phe	Pro	Phe	Asp	Leu	Gln	Asn	Cys	Ser	Met	Lys	Phe	Gly	Ser	Trp	Thr	180	185	190	
Tyr	Asp	Gly	Ser	Gln	Val	Asp	Ile	Ile	Leu	Glu	Asp	Gln	Asp	Val	Asp	195	200	205	
Lys	Arg	Asp	Phe	Phe	Asp	Asn	Gly	Glu	Trp	Glu	Ile	Val	Ser	Ala	Thr	210	215	220	
Gly	Ser	Lys	Gly	Asn	Arg	Thr	Asp	Ser	Cys	Cys	Trp	Tyr	Pro	Tyr	Val	225	230	235	240
Thr	Tyr	Ser	Phe	Val	Ile	Lys	Arg	Leu	Pro	Leu	Phe	Tyr	Thr	Leu	Phe	245	250	255	
Leu	Ile	Ile	Pro	Cys	Ile	Gly	Leu	Ser	Phe	Leu	Thr	Val	Leu	Val	Phe	260	265	270	
Tyr	Leu	Pro	Ser	Asn	Glu	Gly	Glu	Lys	Ile	Cys	Leu	Cys	Thr	Ser	Val	275	280	285	
Leu	Val	Ser	Leu	Thr	Val	Phe	Leu	Leu	Val	Ile	Glu	Glu	Ile	Ile	Pro	290	295	300	
Ser	Ser	Ser	Lys	Val	Ile	Pro	Leu	Ile	Gly	Glu	Tyr	Leu	Val	Phe	Thr	305	310	315	320
Met	Ile	Phe	Val	Thr	Leu	Ser	Ile	Met	Val	Thr	Val	Phe	Ala	Ile	Asn	325	330	335	
Ile	His	His	Arg	Ser	Ser	Ser	Thr	His	Asn	Ala	Met	Ala	Pro	Leu	Val	340	345	350	
Arg	Lys	Ile	Phe	Leu	His	Thr	Leu	Pro	Lys	Leu	Leu	Cys	Met	Arg	Ser	355	360	365	
His	Val	Asp	Arg	Tyr	Phe	Thr	Gln	Lys	Glu	Glu	Thr	Glu	Ser	Gly	Ser	370	375	380	
Gly	Pro	Lys	Ser	Ser	Arg	Asn	Thr	Leu	Glu	Ala	Ala	Leu	Asn	Ser	Ile	385	390	395	400
Arg	Tyr	Ile	Thr	Arg	His	Ile	Met	Lys	Glu	Asn	Asp	Val	Arg	Glu	Val	405	410	415	
Val	Glu	Asp	Trp	Lys	Phe	Ile	Ala	Gln	Val	Leu	Asp	Arg	Met	Phe	Leu				

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420	425	430
Trp Thr Phe Leu Phe Val Ser Ile Val Gly Ser Leu Gly Leu Phe Val		
435	440	445
Pro Val Ile Tyr Lys Trp Ala Asn Ile Leu Ile Pro Val His Ile Gly		
450	455	460
Asn Ala Asn Lys		
465		

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1743 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: both

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 143..1627

(D) OTHER INFORMATION: /product= "ALPHA-6 SUBUNIT"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CGGGTTTTGA TTTCTGAGAA GACACACACG GATTGCAGTG GGCTTCTGAT GATGTCAAGG	60
TTGGATGCAT GTGGCTGACT GATAGCTCTT TGTTTCCAC AATCCTTTC CTAGGAAAAA	120
GGAATCCAAG TGTGTTTTAA CC ATG CTG ACC AGC AAG GGG CAG GGA TTC CTT	172
Met Leu Thr Ser Lys Gly Gln Gly Phe Leu	
1 5 10	
CAT GGG GGC TTG TGT CTC TGG CTG TGT GTG TTC ACA CCT TTC TTT AAA	220
His Gly Gly Leu Cys Leu Trp Leu Cys Val Phe Thr Pro Phe Phe Lys	
15 20 25	
GGC TGT GTG GGC TGT GCA ACT GAG GAG AGG CTC TTC CAC AAA CTG TTT	268
Gly Cys Val Gly Cys Ala Thr Glu Arg Leu Phe His Lys Leu Phe	
30 35 40	
TCT CAT TAC AAC CAG TTC ATC AGG CCT GTG GAA AAC GTT TCC GAC CCT	316
Ser His Tyr Asn Gln Phe Ile Arg Pro Val Glu Asn Val Ser Asp Pro	
45 50 55	
GTC ACG GTA CAC TTT GAA GTG GCC ATC ACC CAG CTG GCC AAC GTG GAT	364
Val Thr Val His Phe Glu Val Ala Ile Thr Gln Leu Ala Asn Val Asp	
60 65 70	
GAA GTA AAC CAG ATC ATG GAA ACC AAT TTG TGG CTG CGT CAC ATC TGG	412
Glu Val Asn Gln Ile Met Glu Thr Asn Leu Trp Leu Arg His Ile Trp	
75 80 85 90	
AAT GAT TAT AAA TTG CGC TGG GAT CCA ATG GAA TAT GAT GGC ATT GAG	460
Asn Asp Tyr Lys Leu Arg Trp Asp Pro Met Glu Tyr Asp Gly Ile Glu	
95 100 105	
ACT CTT CGC GTT CCT GCA GAT AAG ATT TGG AAG CCC GAC ATT GTT CTC	508
Thr Leu Arg Val Pro Ala Asp Lys Ile Trp Lys Pro Asp Ile Val Leu	
110 115 120	
TAT AAC AAT GCT GTT GGT GAC TTC CAA GTA GAA GGC AAA ACA AAA GCT	556
Tyr Asn Asn Ala Val Gly Asp Phe Gln Val Glu Gly Lys Thr Lys Ala	
125 130 135	
CTT CTT AAA TAC AAT GGC ATG ATA ACC TGG ACT CCA CCA GCT ATT TTT	604
Leu Leu Lys Tyr Asn Gly Met Ile Thr Trp Thr Pro Pro Ala Ile Phe	
140 145 150	
AAG AGT TCC TGC CCT ATG GAT ATC ACC TTT TTC CCT TTT GAT CAT CAA	652
Lys Ser Ser Cys Pro Met Asp Ile Thr Phe Phe Pro Phe Asp His Gln	
155 160 165 170	
AAC TGT TCC CTA AAA TTT GGT TCC TGG ACG TAT GAC AAA GCT GAA ATT	700
Asn Cys Ser Leu Lys Phe Gly Ser Trp Thr Tyr Asp Lys Ala Glu Ile	



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Thr Gly Lys Ser  
495

CATATTTGTT CTGCATTCCC TGCCACAAGG AAAGGAAAGC AAAGGCTTCC CACCCAAGTC 1724

CCCCATCTGC TAAAACCCG 1743

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 494 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Leu Thr Ser Lys Gly Gln Gly Phe Leu His Gly Gly Leu Cys Leu  
1 5 10 15  
Trp Leu Cys Val Phe Thr Pro Phe Phe Lys Gly Cys Val Gly Cys Ala  
20 25 30  
Thr Glu Glu Arg Leu Phe His Lys Leu Phe Ser His Tyr Asn Gln Phe  
35 40 45  
Ile Arg Pro Val Glu Asn Val Ser Asp Pro Val Thr Val His Phe Glu  
50 55 60  
Val Ala Ile Thr Gln Leu Ala Asn Val Asp Glu Val Asn Gln Ile Met  
65 70 75 80  
Glu Thr Asn Leu Trp Leu Arg His Ile Trp Asn Asp Tyr Lys Leu Arg  
85 90 95  
Trp Asp Pro Met Glu Tyr Asp Gly Ile Glu Thr Leu Arg Val Pro Ala  
100 105 110  
Asp Lys Ile Trp Lys Pro Asp Ile Val Leu Tyr Asn Asn Ala Val Gly  
115 120 125  
Asp Phe Gln Val Glu Gly Lys Thr Lys Ala Leu Leu Lys Tyr Asn Gly  
130 135 140  
Met Ile Thr Trp Thr Pro Pro Ala Ile Phe Lys Ser Ser Cys Pro Met  
145 150 155 160  
Asp Ile Thr Phe Phe Pro Phe Asp His Gln Asn Cys Ser Leu Lys Phe  
165 170 175  
Gly Ser Trp Thr Tyr Asp Lys Ala Glu Ile Asp Leu Leu Ile Ile Gly  
180 185 190  
Ser Lys Val Asp Met Asn Asp Phe Trp Glu Asn Ser Glu Trp Glu Ile  
195 200 205  
Ile Asp Ala Ser Gly Tyr Lys His Asp Ile Lys Tyr Asn Cys Cys Glu  
210 215 220  
Glu Ile Tyr Thr Asp Ile Thr Tyr Ser Phe Tyr Ile Arg Arg Leu Pro  
225 230 235 240  
Met Phe Tyr Thr Ile Asn Leu Ile Ile Pro Cys Leu Phe Ile Ser Phe  
245 250 255  
Leu Thr Val Leu Val Phe Tyr Leu Pro Ser Asp Cys Gly Glu Lys Val  
260 265 270  
Thr Leu Cys Ile Ser Val Leu Leu Ser Leu Thr Val Phe Leu Leu Val  
275 280 285  
Ile Thr Glu Thr Ile Pro Ser Thr Ser Leu Val Val Pro Leu Val Gly  
290 295 300  
Glu Tyr Leu Leu Phe Thr Met Ile Phe Val Thr Leu Ser Ile Val Val  
305 310 315 320

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Thr	Val	Phe	Val	Leu	Asn	Ile	His	Tyr	Arg	Thr	Pro	Thr	Thr	His	Thr
				325					330					335	
Met	Pro	Arg	Trp	Val	Lys	Thr	Val	Phe	Leu	Lys	Leu	Leu	Pro	Gln	Val
			340					345					350		
Leu	Leu	Met	Arg	Trp	Pro	Leu	Asp	Lys	Thr	Arg	Gly	Thr	Gly	Ser	Asp
		355					360					365			
Ala	Val	Pro	Arg	Gly	Leu	Ala	Arg	Arg	Pro	Ala	Lys	Gly	Lys	Leu	Ala
	370					375					380				
Ser	His	Gly	Glu	Pro	Arg	His	Leu	Lys	Glu	Cys	Phe	His	Cys	His	Lys
385					390					395					400
Ser	Asn	Glu	Leu	Ala	Thr	Ser	Lys	Arg	Arg	Leu	Ser	His	Gln	Pro	Leu
				405					410					415	
Gln	Trp	Val	Val	Glu	Asn	Ser	Glu	His	Ser	Pro	Glu	Val	Glu	Asp	Val
			420					425					430		
Ile	Asn	Ser	Val	Gln	Phe	Ile	Ala	Glu	Asn	Met	Lys	Ser	His	Asn	Glu
	435						440					445			
Thr	Lys	Glu	Val	Glu	Asp	Asp	Trp	Lys	Tyr	Val	Ala	Met	Val	Val	Asp
	450					455					460				
Arg	Val	Phe	Leu	Trp	Val	Phe	Ile	Ile	Val	Cys	Val	Phe	Gly	Thr	Ala
465					470					475					480
Gly	Leu	Phe	Leu	Gln	Pro	Leu	Leu	Gly	Asn	Thr	Gly	Lys	Ser		
				485					490						

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1876 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 73..1581
- (D) OTHER INFORMATION: /product= "ALPHA-7 SUBUNIT"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGCCGAGGC	GCAGCCCGG	GCGACAGCCG	AGACGTGGAG	CGCGCCGGCT	CGCTGCAGCT	60
CCGGGACTCA	ACATGCGCTG	CTCGCCGGGA	GCGTCTGGC	TGGCGCTGGC	CGCGTCGCTC	120
CTGCACGTGT	CCCTGCAAGG	CGAGTTCCAG	AGGAAGCTTT	ACAAGGAGCT	GGTCAAGAAC	180
TACAATCCCT	TGGAGAGGCC	CGTGGCCAAT	GACTCGCAAC	CACTCACCGT	CTACTTCTCC	240
CTGAGCCTCC	TGCAGATCAT	GGACGTGGAT	GAGAAGAACC	AAGTTTTAAC	CACCAACATT	300
TGGCTGCAAA	TGTCTTGGAC	AGATCACTAT	TTACAGTGGA	ATGTGTCAGA	ATATCCAGGG	360
GTGAAGACTG	TTCGTTTCCC	AGATGGCCAG	ATTTGGAAAC	CAGACATTCT	TCTCTATAAC	420
AGTGCTGATG	AGCGTTTGA	CGCCACATTC	CACACTAACG	TGTTGGTGAA	TTCTTCTGGG	480
CATTGCCAGT	ACCTGCCTCC	AGGCATATTC	AAGAGTTCTT	GCTACATCGA	TGTACGCTGG	540
TTCCCTTTG	ATGTGCAGCA	CTGCAAACG	AAGTTTGGGT	CCTGGTCTTA	CGGAGGCTGG	600
TCCTTGGATC	TGCAGATGCA	GGAGGCAGAT	ATCAGTGGCT	ATATCCCCAA	TGGAGAATGG	660
GACCTAGTGG	GAATCCCCGG	CAAGAGGAGT	GAAAGTTTCT	ATGAGTGCTG	CAAAGAGCCC	720
TACCCCGATG	TCACCTTAC	AGTGACCATG	CGCCGCAGGA	CGCTCTACTA	TGGCCTCAAC	780
CTGCTGATCC	CCTGTGTGCT	CATCTCCGCC	CTCGCCCTGC	TGGTGTTCCT	GCTTCTGCA	840

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GATTCCGGGG	AGAAGATTTC	CCTGGGGATA	ACAGTCTTAC	TCTCTCTTAC	CGTCTTCATG	900
CTGCTCGTGG	CTGAGATCAT	GCCCCGAACA	TCCGATTCCG	TACCATTGAT	AGCCCCAGTAC	960
TTCGCCAGCA	CCATGATCAT	CGTGGGCCTC	TCGGTGGTGG	TGACGGTGAT	CGTGCTGCAG	1020
TACCACCACC	ACGACCCCGA	CGGGGGCAAG	ATGCCCAAGT	GGACCAGAGT	CATCCTTCTG	1080
AACTGGTGCG	CGTGGTTCCT	SCGAATGAAG	AGGCCCGGGG	AGGACAAGGT	GCGCCCGGCC	1140
TGCCAGCACA	AGCAGCGGCG	CTGCAGCCTG	GCCAGTGTGG	AGATGAGCGC	CGTGGCGCCG	1200
CCGCCCCGCA	GCAACGGGAA	CCTGCTGTAC	ATCGGCTTCC	GCGGCCTGGA	CGGCGTGCAC	1260
TGTGTCCCGA	CCCCCGACTC	TGGGGTAGTG	TGTGGCCGCA	TGGCCTGCTC	CCCCACGCAC	1320
GATGAGCACC	TCCTGCACGG	CGGGCAACCC	CCCGAGGGGG	ACCCGGACTT	GGCCAAGATC	1380
CTGGAGGAGG	TCCGCTACAT	TGCCAATCGC	TTCCGCTGCC	AGGACGAAAG	CGAGGCGGTC	1440
TGCAGCGAGT	GGAAGTTCGC	CGCCTGTGTG	GTGGACCGCC	TGTGCCTCAT	GGCCTTCTCG	1500
GTCTTCACCA	TCATCTGCAC	CATCGGCATC	CTGATGTCCG	CTCCCAACTT	CGTGGAGGCC	1560
GTGTCCAAAG	ACTTTGCGTA	ACCACGCCTG	GTTCTGTACA	TGTGGAAAAC	TCACAGATGG	1620
GCAAGGCCTT	TGGCTTGGCG	AGATTTGGGG	GTGCTAATCC	AGGACAGCAT	TACACGCCAC	1680
AACTCCAGTG	TTCCCTTCTG	GCTGTACAGC	GTGTTGCTTA	CGGTTTCTTT	GTTACTTTAG	1740
GTAGTAGAAT	CTCAGCACTT	TGTTTCATAT	TCTCAGATGG	GCTGATAGAT	ATCCTTGGCA	1800
CATCCGTACC	ATCGGTCAGC	AGGGCCACTG	AGTAGTCATT	TTGCCCATTA	GCCCCACTGCC	1860
TGGAAAGCCC	TTCGGA					1876

## (2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 502 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met	Arg	Cys	Ser	Pro	Gly	Gly	Val	Trp	Leu	Ala	Leu	Ala	Ala	Ser	Leu
1				5					10					15	
Leu	His	Val	Ser	Leu	Gln	Gly	Glu	Phe	Gln	Arg	Lys	Leu	Tyr	Lys	Glu
				20					25					30	
Leu	Val	Lys	Asn	Tyr	Asn	Pro	Leu	Glu	Arg	Pro	Val	Ala	Asn	Asp	Ser
				35				40				45			
Gln	Pro	Leu	Thr	Val	Tyr	Phe	Ser	Leu	Ser	Leu	Leu	Gln	Ile	Met	Asp
				50			55					60			
Val	Asp	Glu	Lys	Asn	Gln	Val	Leu	Thr	Thr	Asn	Ile	Trp	Leu	Gln	Met
65					70					75					80
Ser	Trp	Thr	Asp	His	Tyr	Leu	Gln	Trp	Asn	Val	Ser	Glu	Tyr	Pro	Gly
				85					90					95	
Val	Lys	Thr	Val	Arg	Phe	Pro	Asp	Gly	Gln	Ile	Trp	Lys	Pro	Asp	Ile
				100				105					110		
Leu	Leu	Tyr	Asn	Ser	Ala	Asp	Glu	Arg	Phe	Asp	Ala	Thr	Phe	His	Thr
				115				120				125			
Asn	Val	Leu	Val	Asn	Ser	Ser	Gly	His	Cys	Gln	Tyr	Leu	Pro	Pro	Gly
				130			135				140				
Ile	Phe	Lys	Ser	Ser	Cys	Tyr	Ile	Asp	Val	Arg	Trp	Phe	Pro	Phe	Asp
145					150					155					160
Val	Gln	His	Cys	Lys	Leu	Lys	Phe	Gly	Ser	Trp	Ser	Tyr	Gly	Gly	Trp

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165					170					175					
Ser	Leu	Asp	Leu	Gln	Met	Gln	Glu	Ala	Asp	Ile	Ser	Gly	Tyr	Ile	Pro
			180					185					190		
Asn	Gly	Glu	Trp	Asp	Leu	Val	Gly	Ile	Pro	Gly	Lys	Arg	Ser	Glu	Arg
		195					200					205			
Phe	Tyr	Glu	Cys	Cys	Lys	Glu	Pro	Tyr	Pro	Asp	Val	Thr	Phe	Thr	Val
	210					215					220				
Thr	Met	Arg	Arg	Arg	Thr	Leu	Tyr	Tyr	Gly	Leu	Asn	Leu	Leu	Ile	Pro
	225					230					235				240
Cys	Val	Leu	Ile	Ser	Ala	Leu	Ala	Leu	Leu	Val	Phe	Leu	Leu	Pro	Ala
				245							250			255	
Asp	Ser	Gly	Glu	Lys	Ile	Ser	Leu	Gly	Ile	Thr	Val	Leu	Leu	Ser	Leu
			260					265						270	
Thr	Val	Phe	Met	Leu	Leu	Val	Ala	Glu	Ile	Met	Pro	Ala	Thr	Ser	Asp
		275					280						285		
Ser	Val	Pro	Leu	Ile	Ala	Gln	Tyr	Phe	Ala	Ser	Thr	Met	Ile	Ile	Val
	290					295					300				
Gly	Leu	Ser	Val	Val	Val	Thr	Val	Ile	Val	Leu	Gln	Tyr	His	His	His
	305					310					315				320
Asp	Pro	Asp	Gly	Gly	Lys	Met	Pro	Lys	Trp	Thr	Arg	Val	Ile	Leu	Leu
				325					330					335	
Asn	Trp	Cys	Ala	Trp	Phe	Leu	Arg	Met	Lys	Arg	Pro	Gly	Glu	Asp	Lys
			340					345					350		
Val	Arg	Pro	Ala	Cys	Gln	His	Lys	Gln	Arg	Arg	Cys	Ser	Leu	Ala	Ser
		355					360					365			
Val	Glu	Met	Ser	Ala	Val	Ala	Pro	Pro	Pro	Ala	Ser	Asn	Gly	Asn	Leu
	370					375					380				
Leu	Tyr	Ile	Gly	Phe	Arg	Gly	Leu	Asp	Gly	Val	His	Cys	Val	Pro	Thr
	385					390					395				400
Pro	Asp	Ser	Gly	Val	Val	Cys	Gly	Arg	Met	Ala	Cys	Ser	Pro	Thr	His
				405					410					415	
Asp	Glu	His	Leu	Leu	His	Gly	Gly	Gln	Pro	Pro	Glu	Gly	Asp	Pro	Asp
			420					425					430		
Leu	Ala	Lys	Ile	Leu	Glu	Glu	Val	Arg	Tyr	Ile	Ala	Asn	Arg	Phe	Arg
		435					440					445			
Cys	Gln	Asp	Glu	Ser	Glu	Ala	Val	Cys	Ser	Glu	Trp	Lys	Phe	Ala	Ala
	450					455					460				
Cys	Val	Val	Asp	Arg	Leu	Cys	Leu	Met	Ala	Phe	Ser	Val	Phe	Thr	Ile
	465					470					475				480
Ile	Cys	Thr	Ile	Gly	Ile	Leu	Met	Ser	Ala	Pro	Asn	Phe	Val	Glu	Ala
				485					490					495	
Val	Ser	Lys	Asp	Phe	Ala										
			500												

## (2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2448 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 265..1773



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(D) OTHER INFORMATION: /product= "BETA-2 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTCCTCCCCC TCACCGTCCC AATTGTATTC CCTGGAAGAG CAGCCGAAA AGCCTCCGCC	60
TGCTCATACC AGGATAGGCA AGAAGCTGGT TTCTCCTCGC AGCCGGCTCC CTGAGGCCCA	120
GGAACCACCG CGGCGGCCGG CACCACCTGG ACCCAGCTCC AGGCGGGCGC GGCTTCAGCA	180
CCACGGACAG CGCCCCACCC GCGGCCCTCC CCCC GGCGGC GCGCTCCAGC CGGTGTAGGC	240
GAGGCAGCGA GCTATGCCCG CGGC ATG GCC CGG CGC TGC GGC CCC GTG GCG	291
Met Ala Arg Arg Cys Gly Pro Val Ala	
1 5	
CTG CTC CTT GGC TTC GGC CTC CTC CGG CTG TGC TCA GGG GTG TGG GGT	339
Leu Leu Leu Gly Phe Gly Leu Leu Arg Leu Cys Ser Gly Val Trp Gly	
10 15 20 25	
ACG GAT ACA GAG GAG CGG CTG GTG GAG CAT CTC CTG GAT CCT TCC CGC	387
Thr Asp Thr Glu Glu Arg Leu Val Glu His Leu Leu Asp Pro Ser Arg	
30 35 40	
TAC AAC AAG CTT ATC CGC CCA GCC ACC AAT GGC TCT GAG CTG GTG ACA	435
Tyr Asn Lys Leu Ile Arg Pro Ala Thr Asn Gly Ser Glu Leu Val Thr	
45 50 55	
GTA CAG CTT ATG GTG TCA CTG GCC CAG CTC ATC AGT GTG CAT GAG CGG	483
Val Gln Leu Met Val Ser Leu Ala Gln Leu Ile Ser Val His Glu Arg	
60 65 70	
GAG CAG ATC ATG ACC ACC AAT GTC TGG CTG ACC CAG GAG TGG GAA GAT	531
Glu Gln Ile Met Thr Thr Asn Val Trp Leu Thr Gln Glu Trp Glu Asp	
75 80 85	
TAT CGC CTC ACC TGG AAG CCT GAA GAG TTT GAC AAC ATG AAG AAA GTT	579
Tyr Arg Leu Thr Trp Lys Pro Glu Glu Phe Asp Asn Met Lys Lys Val	
90 95 100 105	
CGG CTC CCT TCC AAA CAC ATC TGG CTC CCA GAT GTG GTC CTG TAC AAC	627
Arg Leu Pro Ser Lys His Ile Trp Leu Pro Asp Val Val Leu Tyr Asn	
110 115 120	
AAT GCT GAC GGC ATG TAC GAG GTG TCC TTC TAT TCC AAT GCC GTG GTC	675
Asn Ala Asp Gly Met Tyr Glu Val Ser Phe Tyr Ser Asn Ala Val Val	
125 130 135	
TCC TAT GAT GGC AGC ATC TTC TGG CTG CCG CCT GCC ATC TAC AAG AGC	723
Ser Tyr Asp Gly Ser Ile Phe Trp Leu Pro Pro Ala Ile Tyr Lys Ser	
140 145 150	
GCA TGC AAG ATT GAA GTA AAG CAC TTC CCA TTT GAC CAG CAG AAC TGC	771
Ala Cys Lys Ile Glu Val Lys His Phe Pro Phe Asp Gln Gln Asn Cys	
155 160 165	
ACC ATG AAG TTC CGT TCG TGG ACC TAC GAC CGC ACA GAG ATC GAC TTG	819
Thr Met Lys Phe Arg Ser Trp Thr Tyr Asp Arg Thr Glu Ile Asp Leu	
170 175 180 185	
GTG CTG AAG AGT GAG GTG GCC AGC CTG GAC GAC TTC ACA CCT AGT GGT	867
Val Leu Lys Ser Glu Val Ala Ser Leu Asp Asp Phe Thr Pro Ser Gly	
190 195 200	
GAG TGG GAC ATC GTG GCG CTG CCG GGC CGG CGC AAC GAG AAC CCC GAC	915
Glu Trp Asp Ile Val Ala Leu Pro Gly Arg Arg Asn Glu Asn Pro Asp	
205 210 215	
GAC TCT ACG TAC GTG GAC ATC ACG TAT GAC TTC ATC ATT CGC CGC AAG	963
Asp Ser Thr Tyr Val Asp Ile Thr Tyr Asp Phe Ile Ile Arg Arg Lys	
220 225 230	
CCG CTC TTC TAC ACC ATC AAC CTC ATC ATC CCC TGT GTG CTC ATC ACC	1011
Pro Leu Phe Tyr Thr Ile Asn Leu Ile Ile Pro Cys Val Leu Ile Thr	
235 240 245	
TCG CTA GCC ATC CTT GTC TTC TAC CTG CCA TCC GAC TGT GGC GAG AAG	1059
Ser Leu Ala Ile Leu Val Phe Tyr Leu Pro Ser Asp Cys Gly Glu Lys	
250 255 260 265	



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CAAGTAGGGT TTAGCCGGGC CCCATGGTCA CAGACCCCTG GGGGAGGCTT CCAGCTCAGT 2380  
 CCCACAGCCC CTTGCTTCTA AGGGATCCAG AGACCTGCTC CAGATCCTCT TTCCCCACTG 2440  
 AAGAATTC 2448

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 502 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Met Ala Arg Arg Cys Gly Pro Val Ala Leu Leu Leu Gly Phe Gly Leu  
 1 5 10 15  
 Leu Arg Leu Cys Ser Gly Val Trp Gly Thr Asp Thr Glu Glu Arg Leu  
 20 25 30  
 Val Glu His Leu Leu Asp Pro Ser Arg Tyr Asn Lys Leu Ile Arg Pro  
 35 40 45  
 Ala Thr Asn Gly Ser Glu Leu Val Thr Val Gln Leu Met Val Ser Leu  
 50 55 60  
 Ala Gln Leu Ile Ser Val His Glu Arg Glu Gln Ile Met Thr Thr Asn  
 65 70 75 80  
 Val Trp Leu Thr Gln Glu Trp Glu Asp Tyr Arg Leu Thr Trp Lys Pro  
 85 90 95  
 Glu Glu Phe Asp Asn Met Lys Lys Val Arg Leu Pro Ser Lys His Ile  
 100 105 110  
 Trp Leu Pro Asp Val Val Leu Tyr Asn Asn Ala Asp Gly Met Tyr Glu  
 115 120 125  
 Val Ser Phe Tyr Ser Asn Ala Val Val Ser Tyr Asp Gly Ser Ile Phe  
 130 135 140  
 Trp Leu Pro Pro Ala Ile Tyr Lys Ser Ala Cys Lys Ile Glu Val Lys  
 145 150 155 160  
 His Phe Pro Phe Asp Gln Gln Asn Cys Thr Met Lys Phe Arg Ser Trp  
 165 170 175  
 Thr Tyr Asp Arg Thr Glu Ile Asp Leu Val Leu Lys Ser Glu Val Ala  
 180 185 190  
 Ser Leu Asp Asp Phe Thr Pro Ser Gly Glu Trp Asp Ile Val Ala Leu  
 195 200 205  
 Pro Gly Arg Arg Asn Glu Asn Pro Asp Asp Ser Thr Tyr Val Asp Ile  
 210 215 220  
 Thr Tyr Asp Phe Ile Ile Arg Arg Lys Pro Leu Phe Tyr Thr Ile Asn  
 225 230 235 240  
 Leu Ile Ile Pro Cys Val Leu Ile Thr Ser Leu Ala Ile Leu Val Phe  
 245 250 255  
 Tyr Leu Pro Ser Asp Cys Gly Glu Lys Met Thr Leu Cys Ile Ser Val  
 260 265 270  
 Leu Leu Ala Leu Thr Val Phe Leu Leu Leu Ile Ser Lys Ile Val Pro  
 275 280 285  
 Pro Thr Ser Leu Asp Val Pro Leu Val Gly Lys Tyr Leu Met Phe Thr  
 290 295 300  
 Met Val Leu Val Thr Phe Ser Ile Val Thr Ser Val Cys Val Leu Asn  
 305 310 315 320  
 Val His His Arg Ser Pro Thr Thr His Thr Met Ala Pro Trp Val Lys

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325				330				335							
Val	Val	Phe	Leu	Glu	Lys	Leu	Pro	Ala	Leu	Leu	Phe	Met	Gln	Gln	Pro
			340					345					350		
Arg	His	His	Cys	Ala	Arg	Gln	Arg	Leu	Arg	Leu	Arg	Arg	Arg	Gln	Arg
		355					360					365			
Glu	Arg	Glu	Gly	Ala	Gly	Ala	Leu	Phe	Phe	Arg	Glu	Ala	Pro	Gly	Ala
	370					375					380				
Asp	Ser	Cys	Thr	Cys	Phe	Val	Asn	Arg	Ala	Ser	Val	Gln	Gly	Leu	Ala
385					390					395					400
Gly	Ala	Phe	Gly	Ala	Glu	Pro	Ala	Pro	Val	Ala	Gly	Pro	Gly	Arg	Ser
				405					410					415	
Gly	Glu	Pro	Cys	Gly	Cys	Gly	Leu	Arg	Glu	Ala	Val	Asp	Gly	Val	Arg
			420					425					430		
Phe	Ile	Ala	Asp	His	Met	Arg	Ser	Glu	Asp	Asp	Asp	Gln	Ser	Val	Ser
		435					440					445			
Glu	Asp	Trp	Lys	Tyr	Val	Ala	Met	Val	Ile	Asp	Arg	Leu	Phe	Leu	Trp
	450					455					460				
Ile	Phe	Val	Phe	Val	Cys	Val	Phe	Gly	Thr	Ile	Gly	Met	Phe	Leu	Gln
465					470					475					480
Pro	Leu	Phe	Gln	Asn	Tyr	Thr	Thr	Thr	Thr	Phe	Leu	His	Ser	Asp	His
				485					490					495	
Ser	Ala	Pro	Ser	Ser	Lys										
				500											

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1927 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 98..1474
- (D) OTHER INFORMATION: /product= "BETA-3 SUBUNIT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TCGGAACCCC TGTATTTTCT TTTCAAACC CCCTTTTCCA GTGGAATGC TCTGTTGTTA	60
AAAAGGAAGA AACTGTCTTT CTGAACTGA CATCACG ATG CTC CCA GAT TTT ATG	115
Met Leu Pro Asp Phe Met	5
1	
CTG GTT CTC ATC GTC CTT GGC ATC CCT TCC TCA GCC ACC ACA GGT TTC	163
Leu Val Leu Ile Val Leu Gly Ile Pro Ser Ser Ala Thr Thr Gly Phe	
10 15 20	
AAC TCA ATC GCC GAA AAT GAA GAT GCC CTC CTC AGA CAT TTG TTC CAA	211
Asn Ser Ile Ala Glu Asn Glu Asp Ala Leu Leu Arg His Leu Phe Gln	
25 30 35	
GGT TAT CAG AAA TGG GTC CGC CCT GTA TTA CAT TCT AAT GAC ACC ATA	259
Gly Tyr Gln Lys Trp Val Arg Pro Val Leu His Ser Asn Asp Thr Ile	
40 45 50	
AAA GTA TAT TTT GGA TTG AAA ATA TCC CAG CTT GTA GAT GTG GAT GAA	307
Lys Val Tyr Phe Gly Leu Lys Ile Ser Gln Leu Val Asp Val Asp Glu	
55 60 65 70	
AAG AAT CAG CTG ATG ACA ACC AAT GTG TGG CTC AAA CAG GAA TGG ACA	355
Lys Asn Gln Leu Met Thr Thr Asn Val Trp Leu Lys Gln Glu Trp Thr	
75 80 85	

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GAC Asp	CAC His	AAG Lys	TTA Leu	CGC Arg	TGG Trp	AAT Asn	CCT Pro	GAT Asp	GAT Asp	TAT Tyr	GGT Gly	GGG Gly	ATC Ile	CAT His	TCC Ser	403
		90				95							100			
ATT Ile	AAA Lys	GTT Val	CCA Pro	TCA Ser	GAA Glu	TCT Ser	CTG Leu	TGG Trp	CTT Leu	CCT Pro	GAC Asp	ATA Ile	GTT Val	CTC Leu	TTT Phe	451
		105					110						115			
GAA Glu	AAT Asn	GCT Ala	GAC Asp	GGC Gly	CGC Arg	TTC Phe	GAA Glu	GGC Gly	TCC Ser	CTG Leu	ATG Met	ACC Thr	AAG Lys	GTC Val	ATC Ile	499
		120				125					130					
GTG Val	AAA Lys	TCA Ser	AAC Asn	GGA Gly	ACT Thr	GTT Val	GTC Val	TGG Trp	ACC Thr	CCT Pro	CCC Pro	GCC Ala	AGC Ser	TAC Tyr	AAA Lys	547
					140					145					150	
AGC Ser	TCC Ser	TGC Cys	ACC Thr	ATG Met	GAC Asp	GTC Val	ACG Thr	TTT Phe	TTC Phe	CCG Pro	TTC Phe	GAC Asp	CGA Arg	CAG Gln	AAC Asn	595
				155					160					165		
TGC Cys	TCC Ser	ATG Met	AAG Lys	TTT Phe	GGA Gly	TCC Ser	TGG Trp	ACT Thr	TAT Tyr	GAT Asp	GGC Gly	ACC Thr	ATG Met	GTT Val	GAC Asp	643
			170					175					180			
CTC Leu	ATT Ile	TTG Leu	ATC Ile	AAT Asn	GAA Glu	AAT Asn	GTC Val	GAC Asp	AGA Arg	AAA Lys	GAC Asp	TTC Phe	TTC Phe	GAT Asp	AAC Asn	691
					185		190					195				
GGA Gly	GAA Glu	TGG Trp	GAA Glu	ATA Ile	CTG Leu	AAT Asn	GCA Ala	AAG Lys	GGG Gly	ATG Met	AAG Lys	GGG Gly	AAC Asn	AGA Arg	AGG Arg	739
		200				205					210					
GAC Asp	GGC Gly	GTG Val	TAC Tyr	TCC Ser	TAT Tyr	CCC Pro	TTT Phe	ATC Ile	ACG Thr	TAT Tyr	TCC Ser	TTC Phe	GTC Val	CTG Leu	AGA Arg	787
					220					225				230		
CGC Arg	CTG Leu	CCT Pro	TTA Leu	TTC Phe	TAT Tyr	ACC Thr	CTC Leu	TTT Phe	CTC Leu	ATC Ile	ATC Ile	CCC Pro	TGC Cys	CTG Leu	GGG Gly	835
				235				240						245		
CTG Leu	TCT Ser	TTC Phe	CTA Leu	ACA Thr	GTT Val	CTT Leu	GTG Val	TTC Phe	TAT Tyr	TTA Leu	CCT Pro	TCG Ser	GAT Asp	GAA Glu	GGA Gly	883
			250				255						260			
GAA Glu	AAA Lys	CTT Leu	TCA Ser	TTA Leu	TCC Ser	ACA Thr	TCG Ser	GTC Val	TTG Leu	GTT Val	TCT Ser	CTG Leu	ACA Thr	GTT Val	TTC Phe	931
		265					270					275				
CTT Leu	TTA Leu	GTG Val	ATT Ile	GAA Glu	GAA Glu	ATC Ile	ATC Ile	CCA Pro	TCG Ser	TCT Ser	TCC Ser	AAA Lys	GTC Val	ATT Ile	CCT Pro	979
		280				285						290				
CTC Leu	ATT Ile	GGA Gly	GAG Glu	TAC Tyr	CTG Leu	CTG Leu	TTC Phe	ATC Ile	ATG Met	ATT Ile	TTT Phe	GTG Val	ACC Thr	CTG Leu	TCC Ser	1027
					300					305				310		
ATC Ile	ATT Ile	GTT Val	ACC Thr	GTG Val	TTT Phe	GTC Val	ATT Ile	AAC Asn	GTT Val	CAC His	CAC His	AGA Arg	TCT Ser	TCT Ser	TCC Ser	1075
				315				320						325		
ACG Thr	TAC Tyr	CAC His	CCC Pro	ATG Met	GCC Ala	CCC Pro	TGG Trp	GTT Val	AAG Lys	AGG Arg	CTC Leu	TTT Phe	CTG Leu	CAG Gln	AAA Lys	1123
			330					335					340			
CTT Leu	CCA Pro	AAA Lys	TTA Leu	CTT Leu	TGC Cys	ATG Met	AAA Lys	GAT Asp	CAT His	GTG Val	GAT Asp	CGC Arg	TAC Tyr	TCA Ser	TCC Ser	1171
		345					350					355				
CCA Pro	GAG Glu	AAA Lys	GAG Glu	GAG Glu	AGT Ser	CAA Gln	CCA Pro	GTA Val	GTG Val	AAA Lys	GGC Gly	AAA Lys	GTC Val	CTC Leu	GAA Glu	1219
		360				365					370					
AAA Lys	AAG Lys	AAA Lys	CAG Gln	AAA Lys	CAG Gln	CTT Leu	AGT Ser	GAT Asp	GGA Gly	GAA Glu	AAA Lys	GTT Val	CTA Leu	GTT Val	GCT Ala	1267
					380					385					390	
TTT Phe	TTG Leu	GAA Glu	AAA Lys	GCT Ala	GCT Ala	GAT Asp	TCC Ser	ATT Ile	AGA Arg	TAC Tyr	ATT Ile	TCC Ser	AGA Arg	CAT His	GTG Val	1315
				395					400					405		

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AAG AAA GAA CAT TTT ATC AGC CAG GTA GTA CAA GAC TGG AAA TTT GTA	1363
Lys Lys Glu His Phe Ile Ser Gln Val Val Gln Asp Trp Lys Phe Val	
410 415 420	
GCT CAA GTT CTT GAC CGA ATC TTC CTG TGG CTC TTT CTG ATA GTG TCA	1411
Ala Gln Val Leu Asp Arg Ile Phe Leu Trp Leu Phe Leu Ile Val Ser	
425 430 435	
GCA ACA GGC TCG GTT CTG ATT TTT ACC CCT GCT TTG AAG ATG TGG CTA	1459
Ala Thr Gly Ser Val Leu Ile Phe Thr Pro Ala Leu Lys Met Trp Leu	
440 445 450	
CAT AGT TAC CAT TAGGAATTTT AAAAGACATA AGTACTAAAT TACACCTTAG	1511
His Ser Tyr His	
455	
ACCTGACATC TGGCTATCAC ACAGACAGAA TCCAAATGCA TGTGCTTGTT CTACGAACCC	1571
CGAATGCGTT GTCTTTGTGG AAATGGAACA TCTCCTCATG GGAGAAACTC TGGTAAATGT	1631
GCTCATTGT GGTGCCATG AGAGTGAGCT GCTTTTAAAG AAAGTGGAGC CTCCTCAGAC	1691
CCCTGCCTTG GCTTTCCCAG ACATTCAGGG AGGGATCATA GGTCCAGGCT TGAGCTCACA	1751
TGTGGCCAGA GTGCACAAA AGCTGTTGCT ACTTGTTGGA GGAACACCTC CTAGAAGCAG	1811
CAGGCCTCGG TGGTGGGGGA GGGGGGATTC ACCTGGAATT AAGGAAGTCT CGGTGTGCGAG	1871
CTATCTGTGT GGCAGAGCC TGGATCTCCC ACCCTGCACT GGCCTCCTTG GTGCCG	1927

## (2) INFORMATION FOR SEQ ID NO: 16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 458 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Met Leu Pro Asp Phe Met Leu Val Leu Ile Val Leu Gly Ile Pro Ser	
1 5 10 15	
Ser Ala Thr Thr Gly Phe Asn Ser Ile Ala Glu Asn Glu Asp Ala Leu	
20 25 30	
Leu Arg His Leu Phe Gln Gly Tyr Gln Lys Trp Val Arg Pro Val Leu	
35 40 45	
His Ser Asn Asp Thr Ile Lys Val Tyr Phe Gly Leu Lys Ile Ser Gln	
50 55 60	
Leu Val Asp Val Asp Glu Lys Asn Gln Leu Met Thr Thr Asn Val Trp	
65 70 75 80	
Leu Lys Gln Glu Trp Thr Asp His Lys Leu Arg Trp Asn Pro Asp Asp	
85 90 95	
Tyr Gly Gly Ile His Ser Ile Lys Val Pro Ser Glu Ser Leu Trp Leu	
100 105 110	
Pro Asp Ile Val Leu Phe Glu Asn Ala Asp Gly Arg Phe Glu Gly Ser	
115 120 125	
Leu Met Thr Lys Val Ile Val Lys Ser Asn Gly Thr Val Val Trp Thr	
130 135 140	
Pro Pro Ala Ser Tyr Lys Ser Ser Cys Thr Met Asp Val Thr Phe Phe	
145 150 155 160	
Pro Phe Asp Arg Gln Asn Cys Ser Met Lys Phe Gly Ser Trp Thr Tyr	
165 170 175	
Asp Gly Thr Met Val Asp Leu Ile Leu Ile Asn Glu Asn Val Asp Arg	
180 185 190	

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Lys	Asp	Phe	Phe	Asp	Asn	Gly	Glu	Trp	Glu	Ile	Leu	Asn	Ala	Lys	Gly
		195					200					205			
Met	Lys	Gly	Asn	Arg	Arg	Asp	Gly	Val	Tyr	Ser	Tyr	Pro	Phe	Ile	Thr
	210					215					220				
Tyr	Ser	Phe	Val	Leu	Arg	Arg	Leu	Pro	Leu	Phe	Tyr	Thr	Leu	Phe	Leu
225					230					235					240
Ile	Ile	Pro	Cys	Leu	Gly	Leu	Ser	Phe	Leu	Thr	Val	Leu	Val	Phe	Tyr
			245						250					255	
Leu	Pro	Ser	Asp	Glu	Gly	Glu	Lys	Leu	Ser	Leu	Ser	Thr	Ser	Val	Leu
			260					265					270		
Val	Ser	Leu	Thr	Val	Phe	Leu	Leu	Val	Ile	Glu	Glu	Ile	Ile	Pro	Ser
	275						280					285			
Ser	Ser	Lys	Val	Ile	Pro	Leu	Ile	Gly	Glu	Tyr	Leu	Leu	Phe	Ile	Met
	290					295					300				
Ile	Phe	Val	Thr	Leu	Ser	Ile	Ile	Val	Thr	Val	Phe	Val	Ile	Asn	Val
305					310					315					320
His	His	Arg	Ser	Ser	Ser	Thr	Tyr	His	Pro	Met	Ala	Pro	Trp	Val	Lys
			325						330					335	
Arg	Leu	Phe	Leu	Gln	Lys	Leu	Pro	Lys	Leu	Leu	Cys	Met	Lys	Asp	His
			340					345					350		
Val	Asp	Arg	Tyr	Ser	Ser	Pro	Glu	Lys	Glu	Glu	Ser	Gln	Pro	Val	Val
	355						360					365			
Lys	Gly	Lys	Val	Leu	Glu	Lys	Lys	Lys	Gln	Lys	Gln	Leu	Ser	Asp	Gly
	370					375					380				
Glu	Lys	Val	Leu	Val	Ala	Phe	Leu	Glu	Lys	Ala	Ala	Asp	Ser	Ile	Arg
385					390					395					400
Tyr	Ile	Ser	Arg	His	Val	Lys	Lys	Glu	His	Phe	Ile	Ser	Gln	Val	Val
				405					410					415	
Gln	Asp	Trp	Lys	Phe	Val	Ala	Gln	Val	Leu	Asp	Arg	Ile	Phe	Leu	Trp
			420					425					430		
Leu	Phe	Leu	Ile	Val	Ser	Ala	Thr	Gly	Ser	Val	Leu	Ile	Phe	Thr	Pro
	435						440					445			
Ala	Leu	Lys	Met	Trp	Leu	His	Ser	Tyr	His						
	450					455									

## (2) INFORMATION FOR SEQ ID NO: 17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1915 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 87..1583
- (D) OTHER INFORMATION: /product= "BETA-4 SUBUNIT"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCGGCGCTCA	CTCGACCGCG	CGGCTCACGG	GTGCCCTGTG	ACCCACAGC	GGAGCTCGCG	60
GCGGCTGCCA	CCCGGCCCG	CCGGCCATGA	GCGCGCGCC	TTCCCTGGTC	CTTTTCTTCC	120
TGGTCGCCCT	TTGCGGGCGC	GGGAAGTCC	GCGTGCCAA	TGCGGAGGAA	AAGCTGATGG	180
ACGACCTTCT	GAACAAAACC	CGTTACAATA	ACCTGATCCG	CCCAGCCACC	AGCTCCTCAC	240
AGCTCATCTC	CATCAAGCTG	CAGCTCTCCC	TGGCCAGCT	TATCAGCGTG	AATGAGCGAG	300

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AGCAGATCAT	GACCACCAAT	GTCTGGCTGA	AACAGGAATG	GACTGATTAC	CGCCTGACCT	360
GGAACAGCTC	CCGCTACGAG	GGTGTGAACA	TCCTGAGGAT	CCCTGCAAAG	CGCATCTGGT	420
TGCCTGACAT	CGTGCTTTAC	AACAACGCCG	ACGGGACCTA	TGAGGTGTCT	GTCTACACCA	480
ACTTGATAGT	CCGGTCCAAC	GGCAGCGTCC	TGTGGCTGCC	CCCTGCCATC	TACAAGAGCG	540
CCTGCAAGAT	TGAGGTGAAG	TACTTTCCCT	TCGACCAGCA	GAACTGCACC	CTCAAGTTCC	600
GCTCCTGGAC	CTATGACCAC	ACGGAGATAG	ACATGGTCCT	CATGACGCCC	ACAGCCAGCA	660
TGGATGACTT	TACTCCCAGT	GGTGAGTGGG	ACATAGTGGC	CCTCCCAGGG	AGAAGGACAG	720
TGAACCCACA	AGACCCAGC	TACGTGGACG	TGACTTACGA	CTTCATCATC	AAGCGCAAGC	780
CTCTGTTCTA	CACCATCAAC	CTCATCATCC	CCTGCGTGCT	CACCACCTTG	CTGGCCATCC	840
TCGTCTTCTA	CCTGCCATCC	GACTGCGGCG	AGAAGATGAC	ACTGTGCATC	TCAGTGCTGC	900
TGGCACTGAC	ATTCTTCCTG	CTGCTCATCT	CCAAGATCGT	GCCACCCACC	TCCCTCGATG	960
TGCCTCTCAT	CGGCAAGTAC	CTCATGTTCA	CCATGGTGCT	GGTCACCTTC	TCCATCGTCA	1020
CCAGCGTCTG	TGTGCTCAAT	GTGCACCACC	GCTCGCCCAG	CACCCACACC	ATGGCACCCCT	1080
GGGTCAAGCG	CTGCTTCCTG	CACAAGCTGC	CTACCTTCCT	CTTCATGAAG	CGCCCTGGCC	1140
CCGACAGCAG	CCCGCCAGA	GCCTTCCCAG	CCAGCAAGTC	ATGCGTGACC	AAGCCCGAGG	1200
CCACCGCCAC	CTCCACCAGC	CCCTCCAACT	TCTATGGGAA	CTCCATGTAC	TTTGTGAACC	1260
CCGCCTCTGC	AGCTTCCAAG	TCTCCAGCCG	GCTCTACCCC	GGTGGCTATC	CCCAGGGATT	1320
TCTGGCTGCG	GTCCTCTGGG	AGGTTCCGAC	AGGATGTGCA	GGAGGCATTA	GAAGGTGTCA	1380
GCTTCATCGC	CCAGCACATG	AAGAATGACG	ATGAAGACCA	GAGTGTGCTT	GAGGACTGGA	1440
AGTACGTGGC	TATGGTGGTG	GACCGGCTGT	TCCTGTGGGT	GTTTCATGTTT	GTGTGCGTCC	1500
TGGGCACTGT	GGGGCTCTTC	CTGCCGCCCC	TCTTCCAGAC	CCATGCAGCT	TCTGAGGGGC	1560
CCTACGCTGC	CCAGCGTGAC	TGAGGGCCCC	CTGGGTTGTG	GGGTGAGAGG	ATGTGAGTGG	1620
CCGGGTGGGC	ACTTTGCTGC	TTCTTTCTGG	GTTGTGGCCG	ATGAGGCCCT	AAGTAAATAT	1680
GTGAGCATTG	GCCATCAACC	CCATCAAACC	AGCCACAGCC	GTGGAACAGG	CAAGGATGGG	1740
GGCCTGGCCT	GTCCTCTCTG	AATGCCTTGG	AGGGATCCCA	GGAAGCCCCA	GTAGGAGGGA	1800
GCTTCAGACA	GTTCAATTCT	GGCCTGTCTT	CCTTCCCTGC	ACCGGGCAAT	GGGGATAAAG	1860
ATGACTTCGT	AGCAGCACCT	ACTATGCTTC	AGGCATGGTG	CCGGCCTGCC	TCTCC	1915

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 498 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Met	Arg	Arg	Ala	Pro	Ser	Leu	Val	Leu	Phe	Leu	Val	Ala	Leu	Cys	
1			5					10					15		
Gly	Arg	Gly	Asn	Cys	Arg	Val	Ala	Asn	Ala	Glu	Glu	Lys	Leu	Met	Asp
			20					25					30		
Asp	Leu	Leu	Asn	Lys	Thr	Arg	Tyr	Asn	Asn	Leu	Ile	Arg	Pro	Ala	Thr
			35				40					45			
Ser	Ser	Ser	Gln	Leu	Ile	Ser	Ile	Lys	Leu	Gln	Leu	Ser	Leu	Ala	Gln
			50			55				60					
Leu	Ile	Ser	Val	Asn	Glu	Arg	Glu	Gln	Ile	Met	Thr	Thr	Asn	Val	Trp



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65	70	75	80
Leu Lys Gln Glu Trp Thr Asp Tyr Arg Leu Thr Trp Asn Ser Ser Arg	85	90	95
Tyr Glu Gly Val Asn Ile Leu Arg Ile Pro Ala Lys Arg Ile Trp Leu	100	105	110
Pro Asp Ile Val Leu Tyr Asn Asn Ala Asp Gly Thr Tyr Glu Val Ser	115	120	125
Val Tyr Thr Asn Leu Ile Val Arg Ser Asn Gly Ser Val Leu Trp Leu	130	135	140
Pro Pro Ala Ile Tyr Lys Ser Ala Cys Lys Ile Glu Val Lys Tyr Phe	145	150	155
Pro Phe Asp Gln Gln Asn Cys Thr Leu Lys Phe Arg Ser Trp Thr Tyr	165	170	175
Asp His Thr Glu Ile Asp Met Val Leu Met Thr Pro Thr Ala Ser Met	180	185	190
Asp Asp Phe Thr Pro Ser Gly Glu Trp Asp Ile Val Ala Leu Pro Gly	195	200	205
Arg Arg Thr Val Asn Pro Gln Asp Pro Ser Tyr Val Asp Val Thr Tyr	210	215	220
Asp Phe Ile Ile Lys Arg Lys Pro Leu Phe Tyr Thr Ile Asn Leu Ile	225	230	235
Ile Pro Cys Val Leu Thr Thr Leu Leu Ala Ile Leu Val Phe Tyr Leu	245	250	255
Pro Ser Asp Cys Gly Glu Lys Met Thr Leu Cys Ile Ser Val Leu Leu	260	265	270
Ala Leu Thr Phe Phe Leu Leu Leu Ile Ser Lys Ile Val Pro Pro Thr	275	280	285
Ser Leu Asp Val Pro Leu Ile Gly Lys Tyr Leu Met Phe Thr Met Val	290	295	300
Leu Val Thr Phe Ser Ile Val Thr Ser Val Cys Val Leu Asn Val His	305	310	315
His Arg Ser Pro Ser Thr His Thr Met Ala Pro Trp Val Lys Arg Cys	325	330	335
Phe Leu His Lys Leu Pro Thr Phe Leu Phe Met Lys Arg Pro Gly Pro	340	345	350
Asp Ser Ser Pro Ala Arg Ala Phe Pro Pro Ser Lys Ser Cys Val Thr	355	360	365
Lys Pro Glu Ala Thr Ala Thr Ser Thr Ser Pro Ser Asn Phe Tyr Gly	370	375	380
Asn Ser Met Tyr Phe Val Asn Pro Ala Ser Ala Ala Ser Lys Ser Pro	385	390	395
Ala Gly Ser Thr Pro Val Ala Ile Pro Arg Asp Phe Trp Leu Arg Ser	405	410	415
Ser Gly Arg Phe Arg Gln Asp Val Gln Glu Ala Leu Glu Gly Val Ser	420	425	430
Phe Ile Ala Gln His Met Lys Asn Asp Asp Glu Asp Gln Ser Val Val	435	440	445
Glu Asp Trp Lys Tyr Val Ala Met Val Val Asp Arg Leu Phe Leu Trp	450	455	460
Val Phe Met Phe Val Cys Val Leu Gly Thr Val Gly Leu Phe Leu Pro	465	470	475
			480

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Pro Leu Phe Gln Thr His Ala Ala Ser Glu Gly Pro Tyr Ala Ala Gln  
 485 490 495

Arg Asp

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We claim:

1. A stably transfected rodent cell line which has been engineered to express a heterologous protein, said cell line comprising a host cell transformed or transfected with a heterologous nucleic acid molecule comprising a sequence of nucleotides or ribonucleotides that inducibly express an  $\alpha_7$  subunit of a human neuronal nicotinic acetylcholine receptor, wherein said  $\alpha_7$  subunit comprises a sequence of nucleotides selected from the group consisting of:

- (a) a sequence of nucleotides as set forth in SEQ ID No: 11 which encode a human  $\alpha_7$  subunit,
- (b) a sequence of nucleotides that encode a polypeptide as set forth in SEQ ID No: 12;
- (c) a sequence of nucleotides degenerate with the human  $\alpha_7$  subunit polypeptide encoding sequence of (a) or (b).

2. The cell line according to claim 1, wherein the heterologous protein is a functional human neuronal nicotinic acetylcholine receptor.

3. The cell line according to claim 1, further comprising a marker gene, wherein expression of the marker gene indicates expression of the heterologous protein.

10 4. The cell line according to claim 1, wherein the heterologous nucleic acid molecule is confined within an expression vector.

15 5. The stable transfected cell line according to claim 1, wherein the polypeptide of SEQ ID NO: 12 is the only heterologous acetylcholine receptor subunit expressed by the cell.

20 6. A recombinant host cell comprising a heterologous nicotinic acetylcholine receptor that comprises a subunit encoded by a heterologous nucleic acid molecule comprising a sequence of nucleotides or ribonucleotides as set forth in SEQ ID No: 11.

25 7. A recombinant host cell comprising a heterologous nicotinic acetylcholine receptor that comprises a subunit encoded by a heterologous nucleic acid molecule wherein said nucleic acid molecule encodes a polypeptide comprising the sequence of amino acids as set forth in SEQ ID No: 12.

8. The host cell according to claim 7, wherein the polypeptide of SEQ ID NO: 12 is the only heterologous acetylcholine receptor subunit expressed by the cell.

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